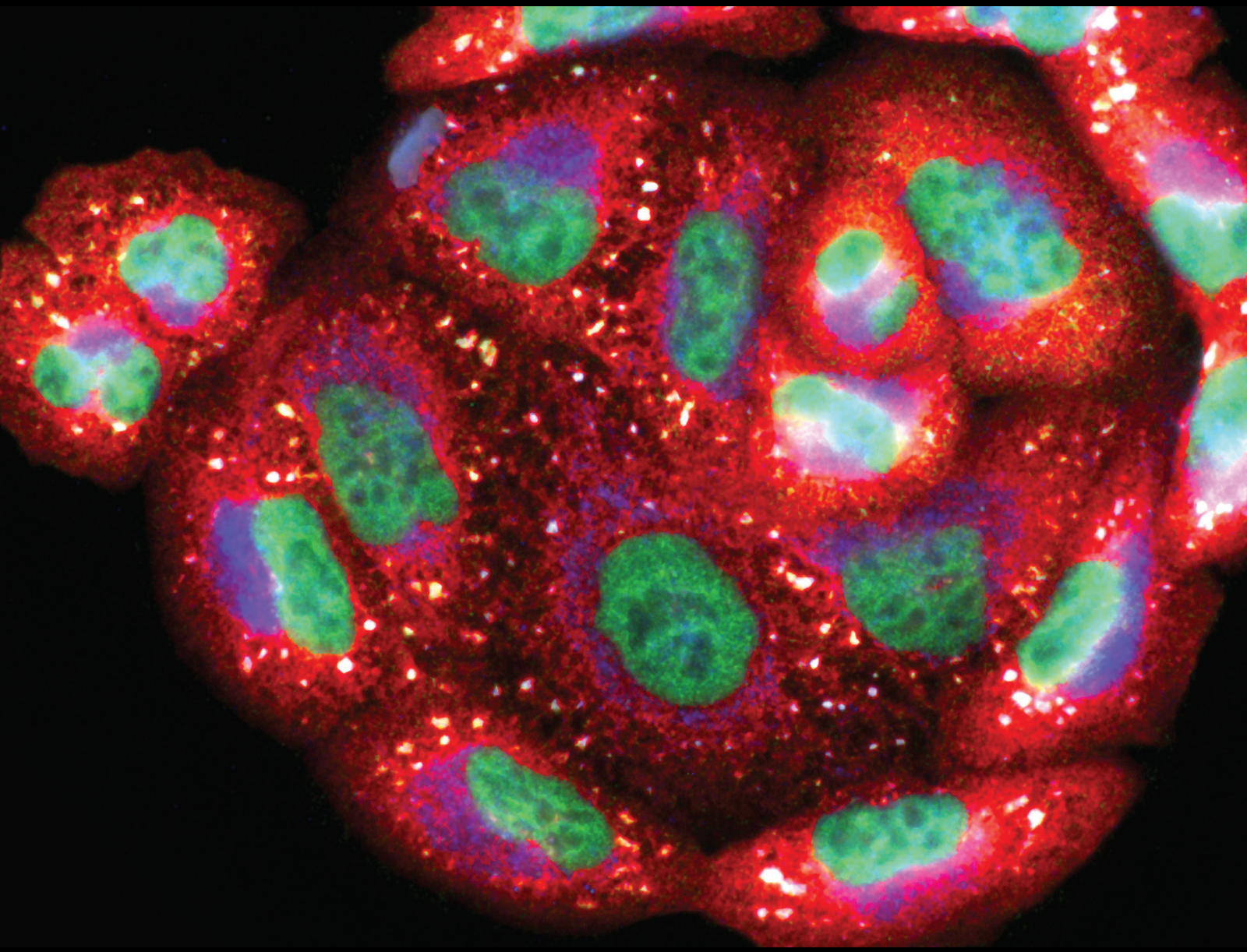
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Pathologies and Aging: Molecular Mechanisms, Therapies and Perspectives 2021

Lead Guest Editor: Claudio Cabello-Verrugio
Guest Editors: Raquel Rodrigues-Díez, Juan Francisco Santibañez, and Daniel Cabrera



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Review Article

Migrasomes: From Biogenesis, Release, Uptake, Rupture to Homeostasis and Diseases

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Migrasomes are migration-dependent membrane-bound vesicular structures that contain cellular contents and small vesicles. Migrasomes grow on the tips or intersections of the retraction fibers after cells migrate away. The process of releasing migrasomes into the extracellular space is named as "migracytosis". After releasing, they can be taken up by the surrounding cells, or rupture and further release their contents into the extracellular environment. Physiologically, migrasomes provide regional cues for organ morphogenesis during zebrafish gastrulation and discard the damaged mitochondria in response to mild mitochondrial stresses. Pathologically, migrasomes are released from podocyte during early podocyte stress and/or damage, from platelets after infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), from microglia/macrophages of the ischemic brain, and from tumor necrosis factor α (TNFα)-activated endothelial cells (ECs); thus, this newly discovered extracellular vesicle is involved in all these pathological processes. Moreover, migrasomes can modulate the proliferation of cancer cell *via* lateral transferring mRNA and protein. In this review, we will summarize the biogenesis, release, uptake, and rupture of migrasomes and discuss its biological roles in development, redox signalling, innate immunity and COVID-19, cardio-cerebrovascular diseases, renal diseases, and cancer biology, all of these highlight the importance of migrasomes in modulating body homeostasis and diseases.

1. Introduction

During the process of cell migration, large vesicles grow on the tips or at the intersections of retraction fibers; eventually, the retraction fibers break, and the pomegranate-like structures (PLSs, referred as "migrasomes"), within a single-limiting membrane, are released into the extracellular space from retraction fibers at the rear of migrating cells; the released PLSs can be directly taken up by the surrounding cells [1, 2]; or they will rupture/disappear and release their contents into the environment in a migration-dependent release process named "migracytosis" [1–4]. The primary function of PLSs is intercellular communication, similar to the mechanisms such as exocytosis and exosome release [1, 2, 5]. This novel extracellular vesicle (EV) has displayed many biological functions. Migrasomes provide regional cues for organ morphogenesis during zebrafish gastrulation [3]. Damaged mitochondria can be discarded *via* migrasomes,

which highlight the essential role of migrasomes in modulating mitochondrial quality control process and redox signalling [6]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) induces platelets to release migrasomes and initiates programmed cell death of platelets in severe coronavirus disease 2019 (COVID-19) [7]. Moreover, tumor necrosis factor α (TNFα) induces the formation of migrasomes in human coronary artery endothelial cells (ECs) [8], parenchymal migrasomes were formed during ischemic brain injury [9], the podocyte-released migrasomes in urine are indicators for early podocyte injury [10], and migrasomes can also modulate cancer cell proliferation *via* lateral transferring mRNA and protein *in vitro* [2]. In this review, we will summarize the current understanding of migrasomes from biogenesis, release, uptake, and rupture to its roles in homeostasis and diseases; and discuss the perspective of migrasomes based on the keywords "migrasome" or "migrasomes" according to the official publishing in PubMed and Web of Science before January 22, 2022.

2. The Discovery of Migrasomes/Migracytosis

The long projections from the surface of cells or the retraction fibrils/fibers from the migrating cells have been observed by Porter [11] and by Taylor [12], respectively; however, retraction fibers have received little attention despite their widespread presence in different cell types [1]. Dr. Yu found "a structure closely resembling an opened pomegranate stood outside a cell" by transmission electron microscopy; the particular image was unusual because there were several of these large structures outside the cell: some of them were empty, some of them had a few vesicles inside, and some of them were packed with vesicles [1, 13]. In 2015, the milestone about PLSs was published in *Cell Research*; Yu's team isolated PLSs by subcellular fractionation *via* density gradient centrifugation and confirmed by transmission electron microscopy [1]. They identified PLS proteins by mass spectrometry analysis and identified tetraspanin-4 (TSPAN4) as the clearest PLS marker by using green fluorescent protein (GFP)-tagged candidate proteins [1]. Besides TSPAN4, wheat-germ agglutinin (WGA), a lectin that binds specifically to sialic acid and N-acetyl-d-glucosamine [14], can also label migrasomes in living cells [15]. Time-lapse imaging revealed that the average lifespan of a PLS derived from normal rat kidney (NRK) cell is about 200-400 min [1, 4]. Using the inhibitor or promoter of migration, they confirmed that the formation of PLSs is migration-dependent (see detail in "The current understanding of migrasomes biogenesis") [1]. Migrasomes are not an artifact caused by culturing cells on a highly rigid surface such as glass or plastic; these PLSs were widely existed in many tissues and cells *in vivo* and *in vitro* [1]. By overexpressing GFP as a tracer for the cytosolic contents in TSPAN4-mCherry-expressing NRK cells, they discovered that the cytosolic components/material and vesicles of unknown origin can actively enter into migrasomes; then, migrasomes and their contents are released into the extracellular space [1, 4]. To their astonishment, they observed that migrasomes left by one cell can be taken up by the surrounding cells [1, 2]. These indicated that migrasomes might play essential roles in modulating intercellular communication.

3. The Basic Features of Migrasomes

The detail methods for visualizing migrasomes by fluorescence microscopy and electron microscopy have been well established by Dr. Chen in Yu's lab [16]. As the novel vesicle structure, we have also summarized the basic differences between migrasomes and exosomes in June, 2020 [17]. The number of smaller vesicles in migrasomes varies greatly; some migrasomes contain up to 300 vesicles, while most contain fewer than 10 [1]. What are these smaller vesicles? The diameters of exosomes are 30-200 nm, which are smaller than that of migrasomes (0.5-3 μm in NRK cells and 1.87 ± 0.18 μm in mice brain) [17]. However, it is unclear whether these smaller vesicles in migrasomes contain exosomes and whether the exosomes reported previously in the literature are partially released by migrasomes [1]. The protein types in migrasomes are not always the same, as that these in NRK cells and in the infarcted brain parenchyma of mice have been shown to be different [1, 9]. Although migrasomes from infarcted brain parenchyma of mice or NRK cells contain RNA-binding proteins, co-staining of F4/80 and RNA did not show RNA signalling within the migrasome either in cerebral cortex or white matter tracts [1, 9]. In contrast, Yu et al. found mRNA in migrasomes from L929 cells (the mouse fibrosarcoma cell line [18]) [2]. Liu et al. have found that miRNA exist in human podocyte cell lines (HPCs)-derived migrasomes, and its miRNA expression profile is different from these in exosomes derived from HPCs [10]. In addition, Antje et al. had identified DNA-interacting proteins in migrasomes from the infarcted brain parenchyma of mice; however, they have not investigated the DNA signalling in these migrasomes [9], and whether the migrasomes of a certain type of cell in a particular state contain DNA is unclear.

Migrasomes extensively distribute in normal human, mouse or rat cells *in vitro*, in cancer cells *in vitro*, and in human, mouse, rat, and zebrafish *in vivo* [1, 3, 6, 9, 10, 19–21] (Figure 1). For example, the differentiation of osteoclasts from murine monocyte-macrophage cell line (RAW 264.7 cells) stimulated by receptor activator of nuclear factor κ-B ligand (RANKL) can induce the formation of vesicle resemble "migrasomes" [21]. Therefore, the location of these PLSs seems to confer them with different biological functions and to investigate their expression patterns in different cells or tissues are of great importance.

4. The Current Understanding of Migrasome Biogenesis

Cell migration is a physically integrated multistep process [22, 23]. To migrate, a cell must acquire a spatial asymmetry enabling it to turn intracellularly generated forces into net cell body translocation; one manifestation of this asymmetry is morphological polarization, i.e., a clear distinction between cell front and rear [22]. An important consequence of polarization is that extension of active membrane processes, which takes place primarily around the cell front

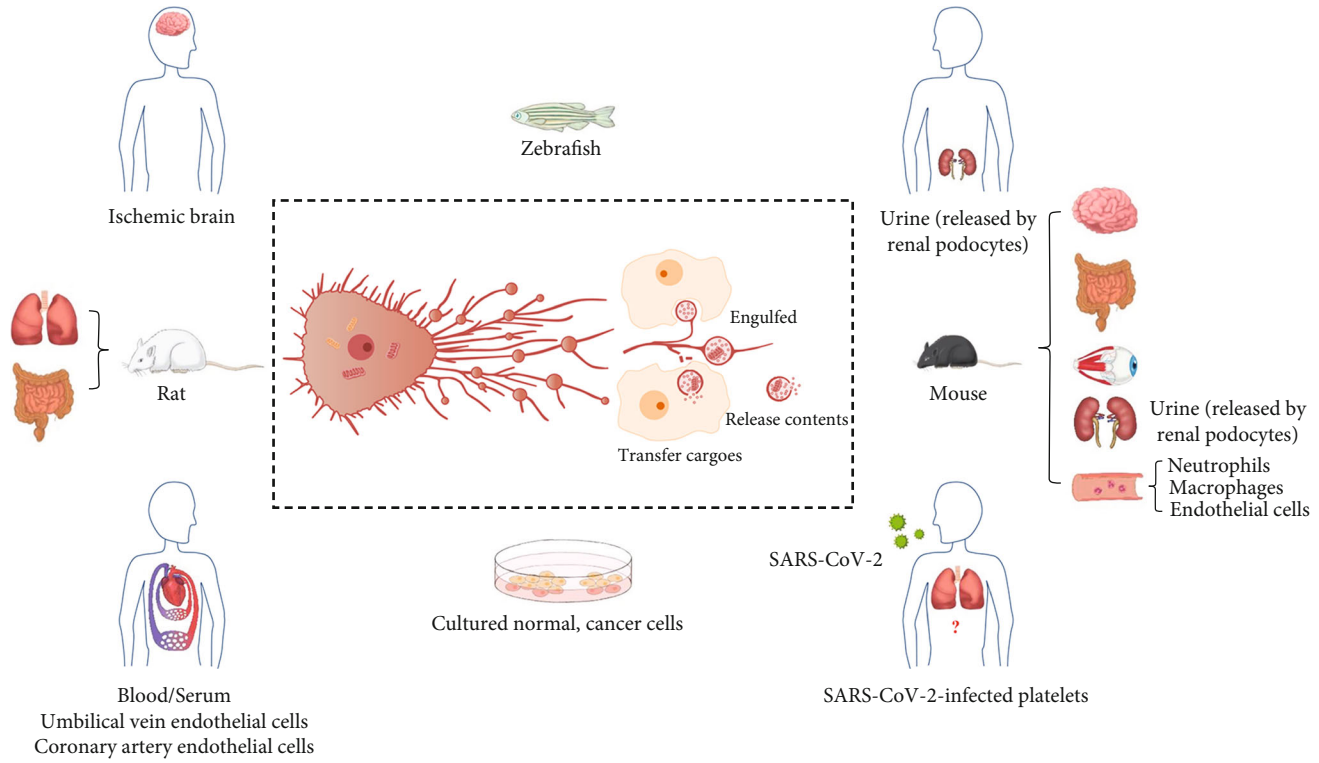

Figure 1: The formation, distribution, and function of migrasomes. Migrasomes are formed and released from the retraction fibers during cell migration; these migration-dependent membrane-bound vesicular structures contain many small vesicles, miRNA, mRNA, proteins, and the swollen mitochondria (the red ones in cell and migrasomes). After releasing, they can be engulfed by the surrounding cells, and transfer their cargoes into the surrounding cells, or rupture and further release their contents into the extracellular environment. Migrasomes are extensively distributed in many cultured cells *in vitro*, such as cancer cells [L929 (mouse fibrosarcoma cell), MDA-MB-231 (human breast cancer), SKOV-3 (human ovarian adenocarcinoma), HCT116 (human colon cancer), SW480 (human adenocarcinoma), MGC803 (human gastric carcinoma), MIACaPa-2 (human pancreatic cancer), and B16 (mouse melanoma)]; the normal human cells [HaCaT (human keratinocyte) and HPC (human podocyte cell)]; the normal mouse cells [primary macrophages, neuron, and embryonic stem cells, RAW 264.7 (mouse monocyte-macrophage), MEF (mouse embryonic fibroblast), and NIH3T3 (mouse embryonic fibroblast)]; and normal rat kidney (NRK) cell. Migrasomes are also found in the key organs of human, mouse and rat *in vivo*. Physiologically, migrasomes serve as chemoattractants to affect organ morphogenesis in zebrafish *in vivo*. Mitocytosis is required for maintaining mitochondrial membrane potential (MMP) and viability in neutrophils in mouse *in vivo*. Migrasomes formed in carbonyl cyanide 3 chlorophenylhydrazone (CCCP)-treated human umbilical vein endothelial cells (ECs) *in vitro* also contribute to maintaining mitochondrial homeostasis. Tumor necrosis factor α (TNFα) induces the formation of migrasomes involved in cell-cell signalization between migrating primary human coronary artery ECs. Pathologically, migrasomes transfer mRNA and protein to modulate the proliferation of cancer cell *in vitro*. Podocyte-released migrasomes in urine serve as an indicator for early podocyte injury in mouse and in human *in vitro*. Migrasomes are in the ischemic brain of mouse and human in vivo, and are involved in the pathological process of ischemic stroke. Migrasomes are detected in human serum samples, and they are released from the platelets in human infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Migrasomes are also found in rat lung and intestine; in mouse intestine and eye, they tend to be located inside cavity, such as blood vessel or pulmonary alveoli; however, their roles in lung injury of severe coronavirus disease 2019 (COVID-19) still need further investigation.

[22]. Therefore, for migration to occur, extend protrusions in the direction of migration must form and then stabilize by attaching to the surroundings [23]. These protrusions can be broad, flat, sheet-like structures, named "lamellipodia," or thin, cylindrical, needle-like projections, named "filopodia" et al.; they are usually driven by actin polymerization, by the cortical expansion mechanism, the Brownian ratchet mechanism, or a combination of these [22, 24]. These protrusions are stabilized by adhering to extracellular matrix (ECM) or adjacent cells *via* the transmembrane migration-promoting receptors, which, as the "feet" of a migrating cell, linked to the actin cytoskeleton *via* adaptors [22, 23]. These adhesions serve as traction sites for migration as the cell moves forward over them [23]. Once the protrusions have become adherent to the substratum, translocation of the cell body forward may occur by myosin interactions with actin filaments, possibly *via* relative movement of adhesion complexes across cortical actin filament "tracks," or the contraction of filaments connecting cell-substratum adhesion complexes with intracellular structures; in either case, the magnitude of traction is greater than the rearward pull on the adhesion complexes [22]. At the rear of the cell, the adhesions are released as the trailing edge detaches from the substratum (here, the magnitude of traction is less than the contraction force), thus allowing net translocation of the cell in the direction of movement and completing a

migratory cycle [22, 25]. Therefore, the polarity is intrinsic to a migrating cell, and the basic cell migration cycle includes extension of a protrusion from cell membrane in the direction of movement, formation of stable attachments near the leading edge of the protrusion, translocation of the cell body forward, release of adhesions, and retraction at the cell rear [22, 23, 25–30].

Time-lapse imaging revealed that formation of migrasomes is likely related to cell migration [1]. Therefore, if migrasome is dependent on migration, modulation events/process involved in migration will influence the formation of migrasome. Indeed, the number or formation of PLSs was largely reduced when reducing cell migration speed by migration inhibitors [1]: the myosin II inhibitor "blebbistatin" [31, 32], and a cell-permeable dynamin inhibitor "dynasore" [33], which has been shown to suppress lamellipodia formation and cancer cell invasion by destabilizing actin filaments [34]. As we have mentioned above, lamellipodia or filopodia formation is usually driven by actin polymerization. The polymerized actin fibers are closely associated with the membrane of some migrasomes [1]. So, what is the function of actin polymerization in migrasomses? Suppressing actin polymerization with cytochalasin B or latrunculin A, or blocking formation of branched actin networks with CK636 (an inhibitor of the Arp2/3 complex, and Arp2/3 complex is an important actin filament nucleator that creates branched actin filament networks required for formation of lamellipodia and endocytic actin structures [35]), reduces the number of PLSs by preventing forming new migrasomes [1]. Thus, actin polymerization is likely required for migrasome formation, either by affecting cell migration as some of these actin polymerization inhibitors can also inhibit migration or by directly involving in migrasome biogenesis [1]. In contrast, the number of migrasomes was increased when accelerating cell migration by different strategies (see detail in below) [1, 4]. Recently, Lu et al. have identified 507 compounds which had significant inhibitory effect on migrasome generation, and 463 out of these 507 hits showed no or less retraction fibers indicating defect of cell migration; this further confirmed that generating migrasome is dependent on migration [36]. Based on migration-dependent biogenesis of PLSs, these PLSs were named as "migrasomes" [1]. Hu et al. has identified "accessible cholesterol"-rich particles released from the macrophage; they are about 30 nm and represent fragments of the plasma membrane that are pulled away and left behind during the projection and retraction of filopodia and lamellipodia [37]. This particle release was abolished when the movement of filopodia/lamellipodia was blocked by blebbistatin or by actin depolymerization (latrunculin A), and their release was increased if the disassembly of focal adhesions (the macromolecular complexes that tether cells to the underlying substrate) was suppressed by FAK inhibitor (CAS 4506-66-5) [37]. Thus, future study focusing on the biological characteristics of these particles and migrasomes is required.

The functional units of cell adhesion typically include cell adhesion molecules/adhesion receptors, the ECM proteins, and the cytoplasmic plaque/peripheral membrane proteins [24]. Among these above, cell adhesion receptors, including members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan (e.g., syndecans) superfamilies, are usually transmembrane glycoproteins that mediate binding interactions at the extracellular surface and determine the specificity of cell-cell and cell-ECM recognition [24]. Integrins are $\alpha\beta$ heterodimers with a large extracellular domain that binds the ECM and links to the actin cytoskeleton by a short cytoplasmic tail [38]. The extracellular domain of integrins determines the binding specificity and recognizes diverse matrix ligands including fibronectin (e.g., $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha4\beta1$), collagen (e.g., $\alpha1\beta1$ and $\alpha2\beta1$), and laminin (e.g., $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$) [38]. Both integrins and TSPAN4 are highly enriched on migrasomes; unlike TSPAN4 that is also abundant on retraction fibers, integrins are only present at very low levels on retraction fibers [1, 4]. Moreover, TSPAN4 was on the upper side, while the endogenous integrin $\alpha5$ and $\beta1$ were enriched on the bottom of migrasomes, and Yu has confirmed that the integrin-enriched regions on migrasomes are not focal adhesions (FAs) [4]. The number of PLSs was increased when accelerating cell migration by knocking down SHARPIN (an endogenous inhibitor of $\beta1$-integrin activation), and fibronectin increased migrasomes number per cell in a dose-dependent manner [1, 36, 39]. GLPG0187, the inhibitor of integrin $\alpha5\beta1$, inhibited the biogenesis of migrasomes in a concentration-dependent manner without cytotoxicity [36]. The integrin $\alpha5$ mRNA levels are much higher than other examined integrins ($\alpha1$, $\alpha2$, $\alpha3$, and $\alpha6$) in TSPAN4-GFP-expressing NRK (NRK-TSPAN4-GFP) cells [4]. Therefore, these cells produced more migrasomes on cover glasses coated with $\alpha5$-pairing fibronectin than with other integrin-pairing laminin 511 or collagen I, and very few migrasomes formed on noncoated cover glasses [4]. Moreover, knockdown of ITGA5 that encodes $\alpha5$ impaired the formation of migrasomes on cells cultured on fibronectin, but not on other ECMs [4]. Overexpressed integrin $\alpha1$ or $\alpha3$ in different cells enhanced migrasome formation, cell spreading, and migration on their corresponding ECM partner protein, but not on other ECM proteins [4]. Therefore, pairing of integrin with its specific ECM partner is a determinant for migrasome formation.

Recently, using chemical screening and RNAi, Yu' team identifies ROCK1 (a positive regulator of microfilament bundle and focal adhesion assembly [40]), rather than ROCK2, as a regulator of migrasome formation [36]. ROCK1 contributes to the formation of migrasomes *via* its role in adhesion to fibronectin to generate a traction force [36]. The role of adhesion on migrasome formation has also been confirmed *in vivo*. The *itgb1b*$^{-/-}$ (encode integrin $\beta1$b) zebrafish embryos formed significantly fewer migrasomes at the gastrulation stages without impaired the speed of cell migration during gastrulation, which implies that integrin $\beta1$b in zebrafish gastrulas most probably regulates migrasome formation by providing adhesion [3]. Saito et al. have evaluated the potential of peptide scaffolds on the forming of migrasomes in cell culture; they found that the peptide interface comprising cell-penetrating peptides (pVEC and R9) and virus fusion peptide (SIV) have superior properties for enabling cell migration and migrasome formation than

fibronectin protein, integrin-binding peptide (RGD), or bare substrate [20]; and these will help us to establish cell model for investigating migrasomes.

The regulatory processes of migrasome biogenesis discussed above were investigated based on migration-dependent characteristics of PLSs. TSPAN4 has been shown as a clearest PLSs marker on migrasomes. However, what is the function of this protein? Indeed, TSPAN4 is a key mediator for migrasome formation [41]: overexpression of 14 (1, 2, 3, 4, 5, 6, 7, 9, 13, 18, 25, 26, 27, and 28) out of the 33 known mammalian TSPANs in NRK cells enhanced migrasome formation, and among these 14 TSPANs, 9 TSPANs (1, 2, 4, 6, 7, 9, 18, 27, and 28) had a strong effect [41]. On the contrary, TSPAN4 deficiency impairs migrasome formation in NRK cells and MGC-803 cells, while knockout of TSPAN4 did not impair migrasome formation in L929 cells [41]. It seems that migrasomes-forming TSPANs have the compensative effect for the loss of TSPAN4, or TSPAN4 totally has no function in migrasome formation in L929 cells. Dynamically, TSPAN4 is recruited to the migrasomes from the retraction fibers during the migrasomal growth phase; in terms of organization, TSPAN4 forms discrete fast-moving puncta that concentrate on the migrasomal surface; and TSPAN4 cannot move from the migrasomes to the retraction fibers once it is recruited to the migrasomes [41]; however, the mechanisms of TSPAN4 nonreturn are not clear. TSPAN4 in migrasomes is about 4 times higher than in retraction fibers [41]. It is easy to think that TSPAN4 alone cannot form a migrasome. The proteins that interact with TSPAN4 might be another breakthrough to answer this question. TSPAN4 belongs to TSPANs family, which includes 33 members in human beings [17]. It has been well known that TSPANs, combined with a set of TSPANs-associated proteins and a high concentration of cholesterol, form a functional unit in cell plasma membranes, named TSPAN-enriched microdomains (TEMs) [17, 41, 42]. Migrasomes were also enriched with other TEMs components, such as integrins and other TSPANs, e.g., TSPAN1, TSPAN2, TSPAN27/CD82, TSPAN28/CD81, and cholesterol, which is about 40-fold in migrasomes relative to retraction fibers [4, 41]. Besides TSPANs and integrins, TEMs component cholesterol is also necessary for migrasome formation as that its formation was impaired when reducing cellular cholesterol levels [41]. The migrasomal membranes were several microns in size, while the typical TEMs are around 100 nm; therefore, the migrasomal membrane is a "TSPANs- and cholesterol-enriched macrodomains (TEMAs)" [41]. Yu's team has established a modified version of the *in vitro* migrasome formation system using the artificially generated giant unilamellar vesicles (GUVs) *via* electrofusion of the proteoliposomes and manually pulling the GUVs membrane by a glass needle; they showed that the biogenesis of migrasomes is mediated by assembling the 100-nm scale TEMs, which exist in the tether membrane, into the micron-scale macrodomains as "TEMAs," and then, these TEMAs swell into the large vesicle-like migrasomal shape [41].

Therefore, TSPANs, cholesterol molecules, and integrins are necessary components for mediating migrasome biogenesis [3, 4, 41]. Mechanistically, active integrins are assembled into puncta on retraction fibers prior to migrasome formation, and the interactions of correct pairing of integrin complexes with its specific ECM partner protein establish the adhesion sites along the retraction fiber, which then serve as platforms for migrasome formation [4, 13]. The integrins on the cell body enable the cell to migrate, whereas the integrins on the migrasomes provide the adhesion for retraction fiber tethering [3, 4]. The mechanical stress exerted along the retraction fibers at the rear of the migrating cell induces the formation of TEMAs *via* triggering the cluster of TSPANs (e.g., TSPAN4 and TSPAN7) and cholesterol molecules; TEMAs enrichment causes the stiffening of the plasma membrane, thus facilitating a new migrasome formation [3, 41, 43].

5. The Current Understanding of the Biological Functions of Migrasomes

5.1. Migrasomes Coordinate Organ Morphogenesis via Serving as Chemoattractants. Migrasomes exist *in vivo*, what are their physiological functions in living organisms? The robust movement of cells during gastrulation of zebrafish embryo, the optical clarity, and out-of-mother development facilitating high-quality imaging make zebrafish embryo as a promising model to visualize, investigate, and characterize endogenous extracellular vesicles (EVs) in real-time and expand our understanding of EVs biology at cellular and systems level [3, 44–47]. The embryonic cells during zebrafish gastrulation generate long projections and migrasomes, which are present in the pockets between the blastodermal margin and the yolk syncytial layer, and in the extracellular pockets of the space between mesendodermal cells [3]. By developing *itgb1b*$^{-/-}$ embryos, maternal zygotic (MZ) tspan7- and MZtspan4a-mutant embryos, TSPAN7 and 4a, and integrin β1b have been confirmed as the key molecules mediating the formation of migrasomes in zebrafish gastrulas [3]. Physiologically, migrasomes act as a source of Cxcl12 during zebrafish gastrulation, and migrasomes are enriched on a large cavity underneath the embryonic shield where they serve as chemoattractants through delivering Cxcl12a for Cxcl12a–Cxcr4b signalling axis to ensure the correct positioning of dorsal forerunner cells vegetally next to the embryonic shield, thereby affecting organ morphogenesis [3].

In addition to zebrafish, migrasome-like structures are also in mouse embryonic stem cells and embryonic fibroblast *in vitro* [1] (Figure 1), while the function of migrasomes in mouse embryonic development has not been identified. Mammalian fertilization comprises sperm migration via the female reproductive tract, biochemical and morphological changes to sperm, and sperm-egg interaction in the oviduct [48]. Motility is one of the most remarkable characteristics of mammalian spermatozoa, while it is not clear whether sperm can produce or release migrasomes; if can, what are the functions of migrasomes in mammalian fertilization?

5.2. Migrasomes Discard the Damaged Mitochondria: An Essential Role in Oxidative Stress. On 2021 May 27, a study

published in *Cell* by Yu's lab has showed that migrasomes contain multiple mitochondria [6]. They found that the oxidative phosphorylation uncoupler carbonyl cyanide 3 chlorophenylhydrazone (CCCP) induces loss of mitochondrial membrane potential (MMP), and generation of high reactive oxygen species (ROS) in mitochondria; subsequently, these damaged mitochondria with an average size of 240 nm and swollen cristae signal their status and subsequently move to the cell periphery through their intrinsic avoidance of binding the inward motor protein dynein, and through globally enhancing the recruitment of KIF5B (the outward motor protein kinesin superfamily protein 5B [49]) to mitochondria; thus, KIF5B selectively binds damaged mitochondria and pulls them to plasma membrane, where myo-19 (myosin-19, an actin-based outer mitochondrial membrane motor, such as propelling mitochondria to filopodia tips [50–52]) tethers mitochondria to cortical actin, which is tightly associated with the plasma membrane [6]. The tips of tubular mitochondria bind to cortical actin and undergo Drp1 (the mitochondrial fission factor [53])-mediated fission, and then, they are sent into migrasomes; these migrasomes containing damaged mitochondrion were referred as "mitosomes"; finally, damaged mitochondria with deleterious mtDNA mutations are selectively disposed of by migrasomes release, a process named as "mitocytosis" [6].

By genetically manipulating the expression of TSPAN4, TSPAN9, dynein, KIF5B and integrin, and by blocking migration using the myosin II inhibitor "blebbistatin" in L929 cells, Jiao et al. found that blocking mitocytosis causes loss of MMP and reduction of spare respiration capacity in cells that are not exposed to mitochondrial stressors, while enhanced mitocytosis improves the loss of MMP and preserves the spare respiration capacity in cells with or without CCCP treatment [6]. Besides CCCP, other mitochondrial stressors, such as deferiprone (a typical iron chelator [54, 55]), antimycin A (a complex III inhibitor [56]), and oligomycin (a selective F1FO-ATPase inhibitor [57, 58]), and starvation can also induce mitocytosis in L929 cells [6]. CCCP treatment also induces mitocytosis in mouse bone marrow-derived macrophages, human pancreatic cancer cells (MIACaPa-2 [59]), and human umbilical vein ECs [6]. Therefore, mitocytosis is a general mechanism that is activated by various mitochondrial stresses in a variety of cells [6].

5.3. Migrasomes in Innate Immunity and COVID-19: Still Long Way to Go. The classical EVs, including exosomes (30-200 nm), microvesicles (approximately 200 nm), and apoptotic bodies (1-2 μm) [17, 60, 61], can convey pathogen molecules that serve as antigens or agonists of innate immune receptors to induce host defence and immunity, or that serve as regulators of host defence and mediators of immune evasion [60]. The functions of EVs on innate immunity are conferred partially by transferring pro- or anti-inflammatory mediators, membrane receptors, enzymes, mRNAs, and noncoding RNAs to the targeting cells and by the interaction of EVs with the complement and coagulation systems [61–65]. Migrasomes are newly discovered EVs and also contain proteins and nucleic acids; what are the functions of migrasomes in regulating innate immunity?

Two studies have shown that macrophages are capable of generating migrasomes [1, 6]. The bone marrow-derived macrophages (BMDMs) derived from TSPAN9$^{-/-}$ mice have significant reduced migrasome numbers and loss of MMP compared to those from wild-type (WT) mice; re-expressing TSPAN9 in TSPAN9$^{-/-}$ macrophages will partially restore the impaired migrasome formation and improve the loss of MMP in cells grown on the untreated migrasome-forming surface, rather than on a hydrophilic surface, which significantly reduces the migration and does not support migrasome formation [6]. These indicated that loss of membrane potential is possibly caused by reduced mitocytosis, rather than by a function of TSPAN9 independent of mitocytosis.

There are migrasomes in human blood, although their origin is not clear [19]. Jiao et al. found that migrasomes were extensively generated by circulating neutrophils in mice, as that around 87% of migrasomes from blood originated from neutrophils [6]. Some neutrophil-derived migrasomes can adhere to vessels in the circulation for a long time, while some are detached quickly after generation [66, 67]. Most importantly, the migrasomes generated by neutrophils contain damaged mitochondria [6]. Migrasome formation by neutrophils from TSPAN9$^{-/-}$ mice is significantly reduced, and the percentage of spleen neutrophils with higher MMP is greatly reduced in TSPAN9$^{-/-}$ mice when compared with the WT, while there is almost no difference in MMP between WT and TSPAN9$^{-/-}$ bone marrow neutrophils, as that bone marrow neutrophils have not yet undergone their long-distance migration; therefore, migrasome/mitocytosis has not yet kicked in; thus, this confirmed that it is migrasome, but not TSPAN9, that contribute to mitochondrial quality control [6].

So, what is the consequence of controlling mitochondrial quality by mitocytosis in immune cells? Jiao et al. showed that the generation and maturation of neutrophils are normal in TSPAN9$^{-/-}$ mice; in contrast, the number of neutrophils is reduced in the spleen from TSPAN9$^{-/-}$ mice compared with WT [6]. When injecting TSPAN9$^{-/-}$ or WT bone marrow neutrophils to WT mice, WT neutrophils significantly outnumber TSPAN9$^{-/-}$ neutrophils after one day's circulation; thus, mitocytosis physiologically contributes to the viability of neutrophils in the circulation [6]. Therefore, disposal of damaged mitochondria *via* releasing migrasomes is essential for keeping circulating neutrophils alive; however, these are limited to the mice in the steady state [6, 67], and using the infectious animal models or patients to explore the functions of migrasomes in other migratory immune cell development and in antiviral innate immunity will be more excited.

With almost two years into the severe coronavirus disease 2019 (COVID-19) pandemic, the impacts of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) go far beyond the suffering and death caused by COVID-19 itself. Scientists around the world are actively investigating SARS-CoV-2 and looking for the effective prevention and control strategies both in modern medicine and in

traditional Chinese medicine. COVID-19 is characterized by pneumonia, lymphopenia, exhausted lymphocytes, and a cytokine storm [68]. Moreover, many patients with COVID-19 present with hypercoagulation and thrombosis [69, 70]. In these patients, platelets become activated and aggregate; these hyperactive platelets activate monocytes, leading to monocyte tissue factor release and thus contributing to the overwhelming thromboinflammation [7, 70–73]. Koupenova et al. revealed that platelets internalize SARS-CoV-2 in an angiotensin-converting enzyme 2 (ACE2)-dependent manner, or in an ACE2-independent manner by attaching to platelet-derived microparticles, and viral internalization leads to rapid digestion, programmed cell death, and release of EVs from platelets, such as microparticles, exosomes, and migrasomes [7]. Rapid platelet death after viral uptake indicates that the platelet milieu does not permit viral replication; this may be protective in immune response; however, the release of platelet contents during dying can be highly prothrombotic or proinflammatory and can lead to dysregulated immune activation [7]. Therefore, these indicated that migrasomes might contribute to thromboinflammation in COVID-19.

Exosomes are also implicated in the pathogenesis of COVID-19; for example, SARS-CoV-2 RNA is present in the exosomal cargo, which suggests that the virus might use the endocytosis route to spread infection [74–81]. Similar to exosomes, migrasomes can also transport cargoes, such as proteins and nucleic acids [2]. Besides in the platelets, brain, blood, and urine of human; in blood, urine, brain, intestine, eye, neutrophils, and macrophages of mouse; and in lung and intestine of rat [1, 6, 7, 9, 10, 19], we do not know the detail distribution and function of migrasomes in human under physiological or pathological conditions. If migrasomes are extensively produced and/or distributed in human body, it seems that they might be more important in SARS-CoV-2 infection and in the pathogenesis of COVID-19. However, this is a bold scientific hypothesis, and it still needs to be carefully verified.

5.4. Migrasomes in Cardio-Cerebrovascular Diseases: Consequence or Contributor? In the ultrathin sections of mouse or rat tissues, migrasomes tend to be present inside cavity such as blood vessel and pulmonary alveoli, for example, in ECs [1]. TNFα induces migrasome formation in ECs, and this formation is highly dependent on cell-cell and cell-ECM interaction, indicating that migrasomes play essential role in the transmission of F-actin-based mechanical forces for proper polarization of adjacent cells and coordination of the cell migration direction [8]. Tropomyosin-1 (a coiled-coil protein that wraps around the actin molecules and provides stability to actin filaments [82]) is a key regulator of TNFα-mediated migrasome formation in ECs, as that angiogenic capacity and migrasome formation were augmented if tropomyosin-1 was knockout in TNFα-activated ECs [8]. CCCP-mediated migrasome formation and mitocytosis in ECs might contribute to mitochondrial quality control process [6]. It has speculated that migrasomes might be a particularly attractive type of signalling vesicles in atherosclerosis due to the high rate of immune cell migration [83].

TSPANs, the key organizers of migrasomes and exosomes, are extensively expressed in hematopoietic and vascular cells and are involved in both physiological and pathological processes related to thrombosis, hemostasis, angiogenesis, and vascular injuries [17, 84]. TSPAN8 expressed in the membrane of exosomes from cancer cells contributed to a selective recruitment of mRNA and proteins into exosomes, including CD106 and CD49d, which were implicated in exosomes-ECs binding and ECs internalization [85]. Subsequently, the exosomal mRNA and/or proteins induce gene expression that suffices for activation of the quiescent ECs, and these exosomes also allow for the survival of EC progenitors (ECP) [85]. We are unclear whether TSPANs in migrasomes have the same influence on vascular homeostasis, or whether the TSPANs-independent effects of migrasomes have the vascular effects.

Migrasomes were formed in F4/80$^+$-microglia/macrophages of ischemic hemispheres of mice that received a standard diet, whereas high salt diet (sodium chloride) enhanced migrasome formation *in vivo*. Sodium chloride can also induce microglial migrasome formation directly *in vitro*, and migrasomes were also detected in postmortem brain tissue of stroke patients [9]. F4/80$^+$-migrasomes are co-localized with NeuN, which is expressed in nuclei and cytoplasm of neurons; this suggests that the two different scenarios are possible: migrasomes might carry off fragments of damaged neurons, thus, fulfilling a "cleavage function"; or migrasomes might incorporate the cytosol of intact neurons, thereby, inducing neuronal death and aggravating ischemic cell damage [9]. It is urgent to know whether migrasome is the consequence or the contributor during ischemic stroke.

5.5. Migrasomes: the Sensitive Indicators for Early Podocyte Stress and/or Damage. Podocyte-derived EVs have received much more attention for nephrologists and others [86–95], and the urinary podocyte-derived EVs are associated with renal injury in systemic lupus erythematosus [95], preeclampsia [91], and metabolic syndrome [90]. Podocytes control glomerular permeability; they have a higher capacity of motility than other renal cells and can release exosomes and migrasomes, while renal tubular cells secrete less migrasomes; these released migrasomes can be detected in human and mouse urine [10]. It should be noticed that migrasomes and exosomes released from podocyte possess different protein and miRNA profiles; for example, the migrasomes contain more PIGK, miR-1303, miR-490-5p, miR-548a, miR-611, and miR-661 than exosomes isolated from the same cultured podocytes [10]. However, it remains unknown the physiological or pathophysiological functions of these migrasomal miRNA and proteins in podocytes.

The secretion of migrasomes in podocytes was strongly enhanced by puromycin amino nucleoside (PAN), lipopolysaccharide (LPS), or a high concentration of glucose (HG) *in vitro* [10]. Release of migrasomes from podocytes is dependent on Rac-1 (Ras-related C3 botulinum toxin substrate1, a member of Rho family of small molecular weight guanosine triphosphate (GTP)-binding proteins/GTPases), as that Rac-1 inhibitor (EHT 1864) dose dependently inhibited LPS-, PAN-, or HG-induced migrasome formation [10,

96–98]. Urinary migrasomes released from podocyte may serve as a more sensitive indicator than proteinuria for podocyte injury, because that increased urinary migrasome number was detected earlier than elevated proteinuria during PAN-induced nephropathy in mice [10, 99]. Furthermore, it is interesting to know whether the migrasomes in urine have the indicative roles for other renal disorders.

5.6. Migrasomes Modulate Cell Proliferation via Lateral Transfer of mRNA and Protein. The intact migrasomes can be engulfed by the surrounding cells; what will happen to the recipient cells after the lateral transfer of cellular contents by migrasomes? In addition to protein, migrasomes also enrich in mRNA [2]. So, when migrasomal proteins and mRNA enter the recipient cells with migrasomes, do the laterally transferred mRNA and proteins have any functional consequence in the recipient cells?

Yu's team chose tumor suppressor *PTEN* (phosphatase and tensin homolog on chromosome 10) as an example, as it was among the most abundant group of mRNAs in migrasomes from L929 cells [2]. *PTEN* was firstly identified as a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer in 1997 [100]. The SUMO1 modification of PTEN modulates tumorigenesis by controlling its association with the plasma membrane; subsequently, PTEN dephosphorylates and converts phosphatidylinositol 3,4,5-triphosphate (PIP3) into phosphtidylinositol 4,5-biphosphate (PIP2) and, thus, antagonizes the phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway [101–104]. Human glioblastoma cell line (U87-MG), breast cancer cell line (MDA-MD-468), and prostate cancer cell line (PC3) fail to express PTEN due to frameshift mutations [2, 105–108]. MDA-MB-468 cells incubated with migrasomes from *Pten* knockout L929 cells displayed no *Pten* protein expression with normal p-Akt levels, while U87-MG, MDA-MD-468, and PC3 cells incubated with purified migrasomes from L929 cells; *Pten* protein was expressed; and Akt phosphorylation was dramatically lowered [2]. Furthermore, migrasomes can induce *Pten* protein expression and reduce the phosphorylation of Akt in recipient cell in dose- and time-dependent manners [2]. Both *Pten* protein and mRNA in migrasomes can transfer into the recipient cells, and *Pten* protein modulates Akt phosphorylation in the recipient cell at earlier time points, while *Pten* mRNA plays a more important role at later time points; in which time, it can translate into *Pten* protein [2]. Migrasome-mediated transfer of *Pten* mRNA and Pten protein can inhibit the proliferation of Pten-deficient MDA-MB-468 cancer cells [2], while the effects of migrasome-mediated cargo transfer in U87-MG and PC3 cells are not clear. In addition, migrasomes are also present in multiple cancer cell types [1], while the functions of migrasomes in these cancers and their roles in tumor metastasis *in vivo* remain to be answered (Figure 1).

6. Discussion

Migrasome is a novel vesicular structure discovered in migrating cells: during cell migration, projections named "retraction fibers" are pulled from the rear end of cells, and large vesicular structures named "migrasomes" grow on the retraction fibers; when the cell migrates away, the retraction fibers break, and migrasomes are left behind [1] (Figure 1). Migrasomes contain many small vesicles, proteins, mRNA, miRNA, and the damaged mitochondria with low MMP and high ROS [2, 6, 10]. The transporting mechanisms of damaged mitochondria to migrasomes are relatively clear [6]. Nevertheless, the origin of small vesicles inside migrasomes and the sorting and transporting mechanisms of these small vesicles, nucleic acids, and proteins into migrasomes remain to be identified [1, 4, 67, 109].

Once detached from cells, migrasomes can be directly taken up by the surrounding cells and transfer their contents into the surrounding cells (Figure 1); according to these, the physiological and pathophysiological functions of migrasomes partially depend on their interaction with the recipient cells [2, 109], although the mechanisms of this interaction and transfer are unclear. It should be considered that the exchange of information between migrasomes and other membranous organelles, such as exosomes, might also influence the functions of migrasomes [110]. There exist essential interorgan communications from the philosophy of traditional Chinese Medicine based on the record in *Huang-Di-Nei-Jing* (also known as "The Yellow Emperor's Canon of Medicine") and in "five-viscus (also known as "five-zang" or "five-organ") correlation theory", and from the philosophy of Western medicine based on modern anatomy, physiology, molecular genetics, and immunology [111]. Similar to other kinds of EVs, migrasomes have a single layer of membrane (phospholipid bilayer structure), which can protect the contents carried by them from being damaged by digestive enzymes in the environment, while their surface have specific adhesion molecules, which can guide them to the correct recipient cells [1, 112]. Migrasomes exist in urine and blood *in vivo* [6, 10, 19], and they have relative long lifespan [1]; there is no doubt that they can travel into the remote organs *via* blood circulation [113]. Considered that migrasomes can be engulfed by the recipient cells or rupture to release their contents into the environment [1–3], hence, they might act as the essential modulators of interorgan communication *in vivo*.

Migrasomes derived from cells can also rupture and release their luminal contents into the environment in a process named "migracytosis" [1–3, 6, 41] (Figure 1). In zebrafish, it is possible that migrasomes have been generated elsewhere by migrating cells, and after breaking from the retraction fibers, they were "washed" to the embryonic shield cavity by moving cells, thus coordinating organ morphogenesis [3]; yet, these still need further investigation. Migrating cells expel dysfunctional mitochondria by releasing migrasomes (a process referred as "mitocytosisis") to protect cells against mitochondrial stressor-induced oxidative damage and maintain mitochondrial homeostasis [6, 114–117]. It is interesting to know what will happen to the damaged mitochondria once transporting into migrasomes and leaving behind the migrating cells. It remains unknown whether mitosomes contain normal mitochondria, and whether mitosomes can be engulfed by the surrounding cells, thus transferring mitochondrial information between cells [6].

Mitochondrial ROS is a key regulator of ECM-degrading metalloproteinases transcription and activation [118]; the pairing of integrins with ECM partner protein is essential for the formation of migrasomes [4], which will release into the ECM after formation [1, 119]; and the released migrasomes can discard the damaged mitochondria with high ROS *via* the process of mitocytosis [6]; hence, the influences of mitocytosis or migrasomes on the status of ECM should also be taken into consideration.

Cell migration is the basic phenomena and fundamental mechanisms of modulating body homeostasis and involved in some human diseases, for example, embryogenesis, wound healing, immune defense, cardio-cerebrovascular diseases, eye diseases, cancer biology, osteoporosis, and the chronic inflammatory diseases, e.g., rheumatoid arthritis and multiple sclerosis [17, 22, 23, 120]. Migrasomes are intrinsically associated with cell migration [6]. Until now, the physiological and pathological functions of migrasomes or its related events have been investigated in zebrafish development model, in mild mitochondrial stresses model of neutrophil and macrophages in mice, in human platelets after internalizing SARS-CoV-2, in ischemic stroke mice model, in PAN-induced nephropathy in mice, and in cancer cell proliferation *in vitro* [2, 3, 7, 9, 10] (Figure 1). Among these diseases above, the contents in migrasomes released from the platelets after SARS-CoV-2 infection have not been determined [7]. Considering the universality of migration in modulating homeostasis and diseases, we speculate that the functions of migrasomes are far more than these examined above. More basic and clinical investigations are needed in the future; for example, the investigation on regulatory mechanisms of migrasome biogenesis, release, uptake, and rupture will help us to further understand the function of these charming vesicles.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review Article

Therapeutic Targets for Regulating Oxidative Damage Induced by Ischemia-Reperfusion Injury: A Study from a Pharmacological Perspective

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Ischemia-reperfusion (I-R) injury is damage caused by restoring blood flow into ischemic tissues or organs. This complex and characteristic lesion accelerates cell death induced by signaling pathways such as apoptosis, necrosis, and even ferroptosis. In addition to the direct association between I-R and the release of reactive oxygen species and reactive nitrogen species, it is involved in developing mitochondrial oxidative damage. Thus, its mechanism plays a critical role via reactive species scavenging, calcium overload modulation, electron transport chain blocking, mitochondrial permeability transition pore activation, or noncoding RNA transcription. Other receptors and molecules reduce tissue and organ damage caused by this pathology and other related diseases. These molecular targets have been gradually discovered and have essential roles in I-R resolution. Therefore, the current study is aimed at highlighting the importance of these discoveries. In this review, we inquire about the oxidative damage receptors that are relevant to reducing the damage induced by oxidative stress associated with I-R. Several complications on surgical techniques and pathology interventions do not mitigate the damage caused by I-R. Nevertheless, these therapies developed using alternative targets could work as coadjuvants in tissue transplants or I-R-related pathologies

1. Introduction

Ischemia-reperfusion (I-R) injury is a cellular phenomenon caused by the interruption of oxygen flow and the consecutive restoration of oxygen concentration, which is known as reperfusion [1]. The reperfusion of ischemic tissues subjected to arterial occlusion causes the formation of a characteristic lesion that accelerates apoptosis and necrosis development [2]. I-R occurs in individuals with multiple pathologies and those receiving an intervention. Thus, it is inevitable in different conditions, such as cardiac, thoracic, and peripheral vascular diseases, and interventions, including major vascular surgery and solid organ transplantation [3–5]. Although the prevalence of ischemia is high, the treatment and preventive strategies for this lesion are not standardized or, simply, not effective enough to resolve damage [6]. Due to the impact of this condition on health systems and its epidemiological distribution, preventive pharmacological strategy is needed urgently. Even when effective therapy is necessary, I-R injury is still poorly understood, and researchers are looking for alternatives or relevant molecular targets that can modulate damage induced by this injury [7].

To date, I-R is characterized by an augmented inflammatory reaction that increases the expression of reactive oxygen species and reactive nitrogen species, which exacerbate tissue damage [8]. Hypoxia-inducible factors (HIF) are oxygen-regulated transcription factors that play important roles in the detection and adaptation of hypoxia [9]. Besides, they act as critical effectors in response to reduced oxygen levels and have a large number of genes under their control [10]. The expression of HIF-1α together with the generation of mitochondrial reactive oxygen species (ROS) is reinforced in response to ischemic oxidative stress [11]. In hypoxia, HIF-1α stabilizes by the accumulation of elevated levels of ROS generated from complex III in the mitochondria [11]. The mechanism behind this is oxidative inactivation of non-heme iron at the catalytic site of the enzyme prolyl hydroxylase [12]. Notably, ROS driven by hypoxia activates NF-kB and other transcription factors such as nuclear factor erythroid 2-related factor 2 (NrF2), which plays a vital role in the regulation of protein transcription involved in antioxidant defense [13]. The mitogen-activated protein kinase (MAPK) pathway has important implications as it interacts with ROS, which leads to a higher expression of vascular endothelial growth factor (VEFG) [14, 15]. The increased expressions of vascular endothelial growth factor (VEGF) and its receptors VEGF-R1 and R2 play a part in the activation of HIF-1α by ROS, and they have fundamental roles in maximizing cell survival [16]. Moreover, ROS activates other intracellular signaling pathways including MAPK, NF-kB, and upstream of MMP [17]. In addition, mitochondrial ROS can enhance damage via different mechanisms, such as mitochondrial permeability induction, ROS-mediated inflammatory and proapoptotic signaling, extracellular remodeling, and primarily oxidative damage in structures and intramitochondrial molecules, which contribute to the development of I-R lesion [18]. The therapeutic value of mitochondrial ROS attenuation in modulating I-R damage to the cell must be emphasized. Hence, effective therapeutic alternatives for ischemic reconditioning and tissue preparation for a possible ischemic event can be developed [19]. Research continues its course. However, certain points must be clarified, and the active principles and crucial receptors that can be an alternative for modulating this phenomenon should be determined [20].

The I-R phenomenon is poorly understood and highly variable between tissues. Although multiple mechanisms are known day by day, there is no effective therapy in clinical phases to date [21]. However, the design of effective therapy is necessary due to the significant relationship between this phenomenon and multiple cardio-obstructive pathologies and surgical procedures [22]. Two main approaches come to light: inflammation and oxidative stress induced by I-R damage [23]. Oxidative stress is very relevant due to the multiple opportunities for damage control. Unfortunately, the mechanisms are not applied. That is the reason for doing this work. Specify and conceptualize the main therapeutic targets towards which the pharmacological designs that allow a resolution of ischemic pathologies should be oriented. Therefore, the current study is aimed at providing ideas and research objectives for resolving oxidative and mitochondrial damage to modulate I-R injury in different tissues.

2. Ischemia-Reperfusion

Over the years, the concept of I-R has been changing and developing, thereby making us closer to discovering or establishing effective therapeutic interventions [7]. In I-R injury, triggering mechanisms begin at the time of arterial blood flow interruption in a tissue or organ, which produces an imbalance of metabolic substrates, leading to hypoxia [18]. I-R is a critical clinical condition, and physicians find it challenging to manage as it requires the preservation of tissue or organ function among individuals with different pathologies or those undergoing surgical procedures [6]. However, in clinical practice, the outcomes after reperfusion in ischemic tissues are far from optimal, and numerous damages are induced to the tissues [24]. As a consequence, reoxygenation is correlated with the exacerbation of local tissue injury and severe local or systemic inflammatory response. This was observed in tissues subjected to I-R, which are comparable with the degree of necrosis observed 24 h after permanent ischemia [25]. Cell dysfunction, damage, and death are associated with the magnitude and duration of ischemia. Therefore, blood flow restoration is still based on injury resolution. However, not all tissues or organs respond similarly to ischemic insult; thus, reperfusion is important to improve cell necrosis [26, 27].

3. Mitochondrial Oxidative Damage in Ischemia-Reperfusion Injury

Mitochondrial oxidative damage is important for the development of I-R, which is directly correlated with mitochondrial ROS and reactive nitrogen species (RNS) formation [28]. In myocardial infarction, the heart requires substantial amounts of energy from phosphates to maintain function and transport [29]. Nevertheless, ATP must be continually synthesized by the oxidative substrate in the mitochondria, thereby increasing the demand for reactive species formation [22]. The inhibition of electron flow along the respiratory chain leads to energy conservation. In addition, limited oxygen supply can inhibit mitochondrial complex IV, which blocks electron transfer to molecular oxygen and reduces ATP concentrations [30]. During the ischemic phase, ATP concentrations are unsuccessfully maintained by glycolysis; hence, the condition further exacerbates, which leads to lactic acid accumulation. Next, intracellular pH decreases. Simultaneously, the Na^+/H^+ antiporter is activated in response to decreased cytosolic hydrogen potential. The cell is overloaded with Na^+, which cannot be pumped out of the cell by Na/K-ATPase. If the ATP concentrations are low due to decreased inner mitochondrial membrane gradient, FOF1-ATPase hydrolyzes ATP to regulate the condition [31]. Due to the inability of the mitochondria to produce significant amounts of ATP, compensatory anaerobic glycolysis occurs as a resolution mechanism. However, paradoxically, a considerable amount of this ATP will be hydrolyzed by FOF1-ATPase

[32]. Concurrently, Na+ can prevent the release of Ca^{2+} by the Na^{+} /Ca^{2+} antiporter, thereby attempting to reverse the process. Calcium could enter the cytosol or even the mitochondria via the reversal of the Na^{+}/Ca^{2+} antiporter mechanism [18]. However, the mitochondrial matrix absorbs Ca^{2+} after the reperfusion process via the uniporter, which then overloads the matrix with this ion. The opening of the mitochondrial permeability transition pore (mPTP) is one of the essential mitochondrial mechanisms in I-R. These pores are strictly linked with mitochondrial ROS and RNS release. Mitochondrial permeability allows ions and solutes with a low weight to freely move between the mitochondrial matrixes [33]. The main concept of mPTP was considered as an in vitro artifact without any pathophysiological significance. A previous study has later supported this notion and confirmed their role in the development of some diseases [34]. ROS are some of the main triggers of mPTP opening by overloading the matrix with high Ca^{2+} concentrations. However, there are other factors and molecules implicated in reperfusion. One of them is the influx of oxygen in anoxic cells, which leads to the formation of free radicals, a consequence of respiratory chain inhibition [34]. Almost all free radicals may be produced via the activation of xanthine oxidase. This enzyme is activated in hypoxia during ischemia [35].

In addition to cellular phosphate and depleted adenine nucleotide levels, which are commonly correlated with the ischemia process, high Ca^{2+} concentrations and oxidative stress conditions can activate mPTP [35]. During the reperfusion phase, the pH returns to preischemic insult values. This phenomenon is attributed to the activity of Na^{+}/H^{+} antiporter that grants the release of lactic acid, which makes mPTP relevant and facilitates its full ability to exhibit its effect [36].

4. Oxidative Molecular Mechanisms Involved in Ischemia-Reperfusion Injury

There are complementary processes that are directly or indirectly correlated with mitochondrial oxidative stress and that play an essential role in the development of I-R injury [37]. Events, such as increased cations at the cytosolic level, mitochondrial injury, formation of oxidative and nitrosative species, transcriptional reprogramming, apoptosis activation processes, autophagy, necrosis, inflammation, immunity-mediated injury, endothelial injury, activation of ferroptosis, and the nonreflux phenomenon, are triggered or enhanced by blood flow obstruction and restoration [38].

4.1. Calcium Overload. Calcium overload and cytosolic cation increment are the initial mechanisms activated after the start of ischemia. All tissues and cells affected by this condition become dependent on anaerobic glycolysis ATP supply [39]. However, as an alternative for restoring pH to normal levels, some anticarriers including Na^{+}/H^{+} are activated to address the accumulation of cytosolic Ca^{2+}. Nevertheless, the expression of cytosolic Ca^{2+} is even higher during reperfusion, when the removal of H^{+} ions of extracellular origin paradoxically raises the proton gradient, thereby accelerating the proton exchange function [40]. All these events and alterations as well as high Ca^{2+} concentrations activate different pathways involved in I-R-induced cell death. Pumping up Ca^{+} directly to the mitochondria via Ca^{2+} uniporters is a mechanism that can help cells manage Ca^{+} overload [41].

4.2. Formation of Reactive Oxygen and Nitrogen Species in Mitochondria. ROSs are normally produced in the mitochondria, endoplasmic reticulum, plasma membrane, and cytoplasm during physiological metabolic processes [42]. ROS and RNS production in the cell starts with the reduction of oxygen and nitrogen levels, which are extremely basic and simple reactions. However, they are extremely important in cell function [43]. Generally, the mitochondria are the main source of cellular oxidative and nitrosative stress. Nonetheless, study results that reinforce this argument, particularly in nitrosative stress and heart disease, must be further validated [44, 45]. The mitochondria are involved in reactive species formation, with production directly involved with cytosol reactive species concentrations [46]. Moreover, complexes I and III of the electron transport chain are involved in ROS formation along with NAD^{+}-linked oxidoreductases in the mitochondrial matrix. This notion has been reviewed and presented in several studies [42, 47, 48]. The reactive species correlated with the mitochondria and oxidative and nitrosative damage are superoxide molecules, hydrogen peroxide, hydroxyl radicals (-OH), nitric oxide (NO), nitroxyl anion, nitrosonium cation (NO^{+}), and peroxynitrite (ONOO-) [49, 50].

4.3. Ferroptosis in I-R Phenomenon. Ferroptosis is a type of cell death that is an alternative to apoptosis. It is characterized by the accumulation of iron-dependent lipid hydroperoxides at alarming levels. Moreover, it cannot be inhibited by factors associated with other known types of cell death [51]. Hence, it is morphologically, biochemically, and genetically different from other types of cell death, and it is involved in various pathological events in which I-R is not an exemption [52]. Further, it is one of the relevant oxidative pathways that could modulate I-R damage due to its close association with some oxidative components in this pathology [53]. Lipid ROS accumulation, which leads to oxidation and antioxidation activity mechanism via toxic lipid peroxidation, is a principal ferroptosis pathway that could be correlated with I-R [54, 55]. As an initiation mechanism, glutathione peroxidase (GPx) has an important antioxidant role in ferroptosis during reperfusion. Oxygenated blood promotes the stimulation of enzyme activity, primarily its isoform 4 (GPX4), which has the capabilities of a cytosolic antioxidant enzyme. This phenomenon then modulates the substrates of the lipoperoxide pathways such as H_2O_2, small hydroperoxides, and phospholipids that are inserted in the biomembranes [56, 57]. By contrast, arachidonic acid contains phosphatidylethanolamine, which plays a key role in the ferroptosis cell death signaling. Hence, it is a crucial target for modulating this oxidizing process [57–59].

5. Antioxidant Enzymes in I-R

Researchers are examining the safest and most effective therapies for regulating I-R damage. However, this tissue pathology is characterized by excessive oxidative damage that is challenging to resolve because free radicals can drive cells via different routes of cell death and subsequent necrosis [20]. Nevertheless, in recent years, a previous study about ROS has shown that these molecules are involved in different pathological processes closely correlated with I-R [60]. Therefore, antioxidant activity is the main therapeutic target of most pharmacological therapies to address this phenomenon [60]. However, not all therapeutic approaches have shown conclusive or favorable results. Thus, it is constantly necessary to make updates on antioxidant therapy in this event to improve the resolution of this pathophysiological condition [61]. The scientific community accepts the role of ROS and other important free radicals, such as superoxide radical (O2), formed by adding extra electrons in an oxygen molecule (OH), which is created from O_2 via the interaction of H_2O catalyzed by transition metals including iron in I-R [62]. Oxygen radicals can be formed by the action of singlet oxygen, which commonly occurs in ischemic tissues [63]. The eukaryotic cell has a defense system similar to that of enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase, which inhibit reactive species formation [64, 65] For example, the concentrations of SOD1, SOD2, CAT, and GSH-Px decrease in I-R [66, 67]. Another important antioxidant complication is the reduction of GSH in the ischemic myocardium by buthionine sulfoximine, a cellular inhibitor of GSH, thereby making the tissue more susceptible to reperfusion damage [68]. One of the main concerns when choosing antioxidant enzymes as possible therapeutic targets is that the enzyme activity and the average concentration in tissue and the relationship they have in ischemia-reperfusion damage have not yet been correctly described or have not been conclusive [69].

6. Therapeutic Targets in Oxidative Damage

A detailed review of the therapeutic targets that can be a receptor for some antioxidant drugs should be performed. This allows redirecting research to the establishment of therapeutic alternatives that can have more interesting effects [70]. Calcium overload, which triggers the formation of reactive species in the mitochondria, and MPTP opening, which is involved in the release of $Ca2^+$ from the mitochondrial matrix to the cytosol, are relevant [71]. Regulating the ferroptosis process, one of the key pathways in nonapoptotic death that is strictly correlated with cellular antioxidant capacity could be an interesting alternative for different pathologies involving I-R [64]. Undoubtedly, the modulation of ROS/RNS in the cytosol, which can prevent all types of cell damage, is a strategy that remains undiscovered due to a large number of possibilities [72]. Notably, future research must focus on the different types of tissues, variations in I-R injury, and modulating strategies.

6.1. Mitochondrial Receptors. Alternatives for modulating I-R damage should be identified. Oxygen depletion after ischemia is correlated with the inhibition of mitochondrial respiratory chain electron transport and the consecutive decrease in ATP levels, which leads to failure in Na^+/K^+ pump and Ca^{2+} accumulation [73, 74]. The electron transport chain can be the primary target of the mitochondria. This is confirmed by five main types of enzyme complexes, which are as follows: NADH-CoQ reductase, succinate-COQ reductase, CO-Q-cytochrome c reductase, cytochrome c oxidase, and ATP synthase, which are known in that order as complexes I–V and are all integrated into the inner mitochondrial membrane [75, 76]. Concurrently, ubiquinone and cytochrome c are the two freely diffusible molecules implicated in electron transfer between complexes previously mentioned [77]. Various signaling pathways, which are essential to normal cell function, require ROS activity from hydrogen peroxide, hydroxyl radicals, and superoxide anions. The main keys for triggering the formation of ROS are found in complexes I and III [78]. NADH commonly binds to the complex and promotes electron transfer flavin mononucleotide (FMN). Reduced levels of flavin decrease O_2 superoxide concentrations and promote proton transfer that conducts ATP synthesis [78]. Mitochondrial complex II is the only enzyme that is part of both the Krebs cycle and electron transport chain. Succinate, which is oxidized to fumarate via the action of adenine flavin, mediated by the dinucleotide cofactor (FAD), is involved in this reaction [79]. Undoubtedly, mitochondrial complex II is a central modulator in metabolic and respiratory adaptation in response to different stimuli and abnormalities. Thus, it is a key receptor in the modulation of oxidative damage in I-R [80]. Previous reports have shown an overlap in respiratory complex II and mKATP channel agonists that can activate it. The association between mKATP and respiratory chain complexes has shown a correlation between complex II and decreased ROS production [81].

The complex III Q-cytochrome c reductase molecule, which is implicated in the addition of four protons to the intermembrane space, is a significant site for ROS productions. The free radical ubisemiquinone leads electrons to oxygen, and this reaction results in a superoxide ion formation process that is enhanced by complex III inhibition [82]. Cytochrome c oxidase, better known as complex IV, mediates O_2 reduction from H_2O molecules by transferring four protons from the matrix into the intermembrane space, thereby increasing the electrochemical gradient and then entering as part of the intermediaries to this reaction [83, 84]. ATP synthase complex V promotes oxidative phosphorylation and induces ATP synthesis resulting in ATP formation [85]. Therefore, failure in the activity of this complex leads to inefficiency and dysregulation of mitochondrial function [86]. Complexes I and III are the principal targets because small amounts of free radicals are correlated with oxidative damage induction, mainly during hypoxia or ischemia. The inhibitors or modulators of these two molecules could manage hypoxic or ischemic conditions [87]. Interestingly, modulating mPTP opening is another interesting point for preventing ROS damage or even necrosis. This

complex is a crucial effector in the cell death pathway. In addition, the activation of the mPTP function is the first step in the mitochondrial intrinsic necrosis pathway, leading to mitochondrial permeability transition and loss of inner mitochondrial potential [88]. Several pathways lead to the opening of mPTP. As a protein complex, they must interfere in one of its subunits, cyclophilin D (CyD), an essential modulator of mPTP. This makes it a key target for preventing cell death due to necrosis [89, 90]. A recent study focuses on the identification of novel compounds that can inhibit mPTP opening without any modulation of the CyD [91]. Mitochondrial ATP-sensitive potassium channels (mKATP) are opened after ischemia as a resolution measure, thereby modifying the activation of mPTP and delaying apoptosis. In addition, nonmitochondrial KATP can provide protective effects by promoting blood flow and excessive production of substrates [92]. These complexes promote the blocking of mitochondrial respiration and membrane disruption during diseases. Further, they are considered the primary cause of cell death in myocardial infarction I-R [93–95]. The mitochondrial antioxidant manganese SOD (MnSOD) expression is one of the objectives for modulating its dismutase scavenging function in superoxide radical O_2 affecting several cell compartments. These are correlated with the pathophysiology of I-R, with the endoplasmic reticulum being sensitive to ROS, thereby making it responsible for maintaining calcium homeostasis [96, 97]. By contrast, autophagy is a crucial modulation target, which is responsible for cell recycling [98]. Previous studies have shown that this event contributes to the processes of cellular damage, and the key molecules are Beclin 1, mTOR, and PI3K [99, 100]. The mitochondria are important in pathological processes. To date, there is sufficient evidence about the morphological differences between the mitochondria, and that they are structurally and physiologically distinguished even in the same tissue [101] (Figure 1).

7. Molecular Targets for Ischemia in Different Tissues

Although the I-R phenomenon has many similarities in different tissues, the lack of oxygen is the leading cause of cellular imbalance. On the other hand, it is necessary to highlight the differential characteristics between tissues, mainly the critical therapeutic targets that will elucidate pathophysiological mechanisms and the design or implementation of new therapeutics or interventions. In this work, we selected some groups of tissues most affected by this phenomenon and try to highlight the molecular targets.

7.1. Myocardium. The myocardium is an I-R susceptible tissue after epicardial coronary artery occlusion. The hypoperfused myocardial zone during myocardial infarction is a risk zone for oxidative damage and inflammation [102]. Clinical and preclinical research has shown a large number of cardioprotective agents, with mechanisms ranging from calcium overload to oxidative stress modulation. However, targeted therapy remains a challenge that has not been addressed altogether [103]. Calcium (Ca^{2+}) released from the sarcoplasmic reticulum (SR) is important for excitation-contraction (E-C) coupling. The mitochondria, the major source of energy in the form of ATP, which is required for cardiac contractility, are closely interconnected with the SR, and Ca^{2+} is essential for the optimal function of these organelles. However, Ca^{2+} accumulation can impair mitochondrial function, leading to reduced ATP production and increased release of ROS. The calcium (Ca^{2+}) released by the SR is essential for cardiac excitation and contraction. ATP from the mitochondria is the main source of energy for the myocardial contraction process. However, the accumulation of mitochondrial Ca^{2+} affects the functioning of this organelle, which significantly decreases ATP and increases the formation of ROS [104]. Oxidative stress is directly associated with heart failure. Some studies have validated the role of Ca^{2+} in the development of this event, and it was found to be closely related to mitochondrial dysfunction [105]. Notably, there are two ways of releasing Ca^{2+} accumulating in the mitochondria of the cardiac cell, which are as follows: via type 2 ryanodine receptors RyR2 and type 2 inositol 1,4,5-triphosphate (IP3R2) receptors [106, 107] (Figure 2).

7.2. Hepatic I-R. The liver is extremely sensitive to oxidative damage caused by I-R. Therefore, blood flow must be restored to prevent or slow down cell death [108]. Some studies have reported the importance of ischemic preconditioning for the management of this pathology and the role of lipoperoxidation modulation in this mechanism. This explains why peroxidation signaling pathways are relevant in reducing this condition [109]. By contrast, it is important to identify the role of peroxisome proliferator-activated receptor-gamma (PPAR-γ). That is, it inhibits the production of ROS in a pre- and posttransductional method, via the FAM3A complex and noncoding RNA axis, as reported by several workgroups [110]. In addition, the other important targets for modulating oxidative damage are metalloproteinases and malondialdehyde, which are the enzyme complexes involved in the cellular oxidative process [111, 112] (Figure 3).

7.3. Renal Tissue. The kidney is a specific organ that can be affected by I-R, which could lead to irreversible kidney injury. However, renal occlusion is inevitable during transplantation. That is why there are countless models for this phenomenon [113]. These advancements are crucial in understanding the pathophysiology of renal I-R, and they propose some therapeutic targets that can improve management. It is important to provide an overview of the possible therapies and receptors that can reduce oxidative damage in the kidneys [113, 114]. Similar to other organs, ROS plays a fundamental role in oxidative stress, which changes mitochondrial oxidative phosphorylation, ATP depletion, an increase of intracellular calcium, and activation of membrane phospholipid proteases, processes that could have results as a therapeutic alternative [115]. The interesting molecules to modulate the damage induced by renal I-R are more aimed at increasing the expression of antioxidant enzymes or their activity to offer a resolution of oxidative

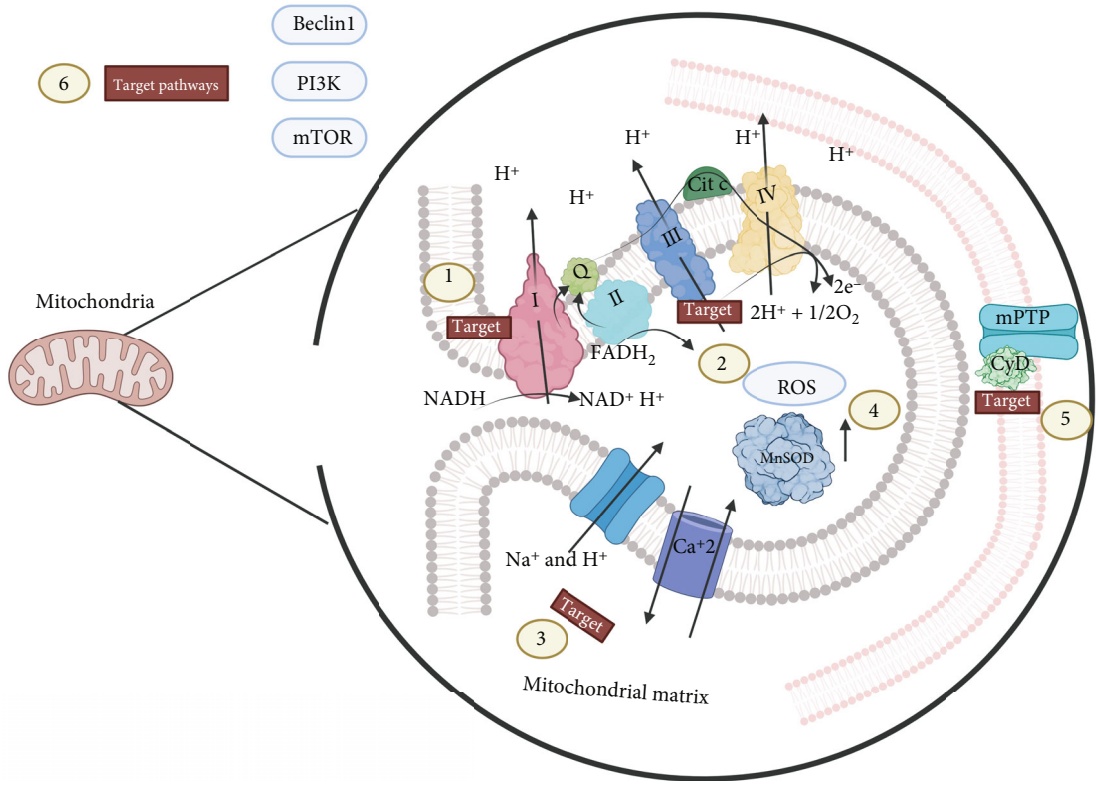

FIGURE 1: Mitochondrial targets for modulating oxidative damage induced by ischemia-reperfusion. The electron transport chain mainly in complexes I (1) and III (2) is the target of interest for reducing the formation of reactive oxygen species in the mitochondrial matrix as well as the activation of Na^+/H^+ antiporters and calcium saturation (3) in the matrix along with the increased expression of antioxidant enzymes such as MnSOD (4) that carry out free radicals. In the mitochondria membrane, the blocking of MPTP by cyclophilin (CyD) is also essential (5), thereby preventing ROS from leaving the mitochondria to the cytosol. Beclin 1, PI3K, and mTOR (6), which are alternatives of interest for blocking mitochondrial damage, are some pathways that have good outcomes for reducing such damage.

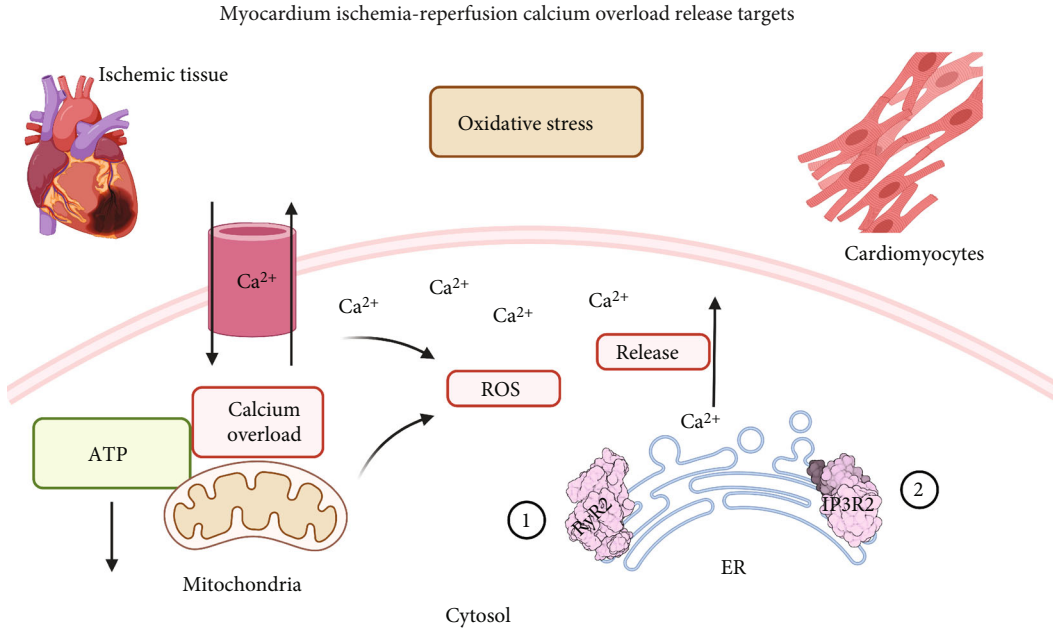

FIGURE 2: Calcium overload release targets. There are two principal ways of releasing Ca^{2+} accumulating in the mitochondria of the cardiomyocytes due to mitochondrial ROS formation. The first one is via type 2 ryanodine receptors RyR2 (1) and type 2 inositol 1,4,5-triphosphate (IP3R2) receptors (2). A mechanism that reduces ROS and Ca^{2+} cell damage.

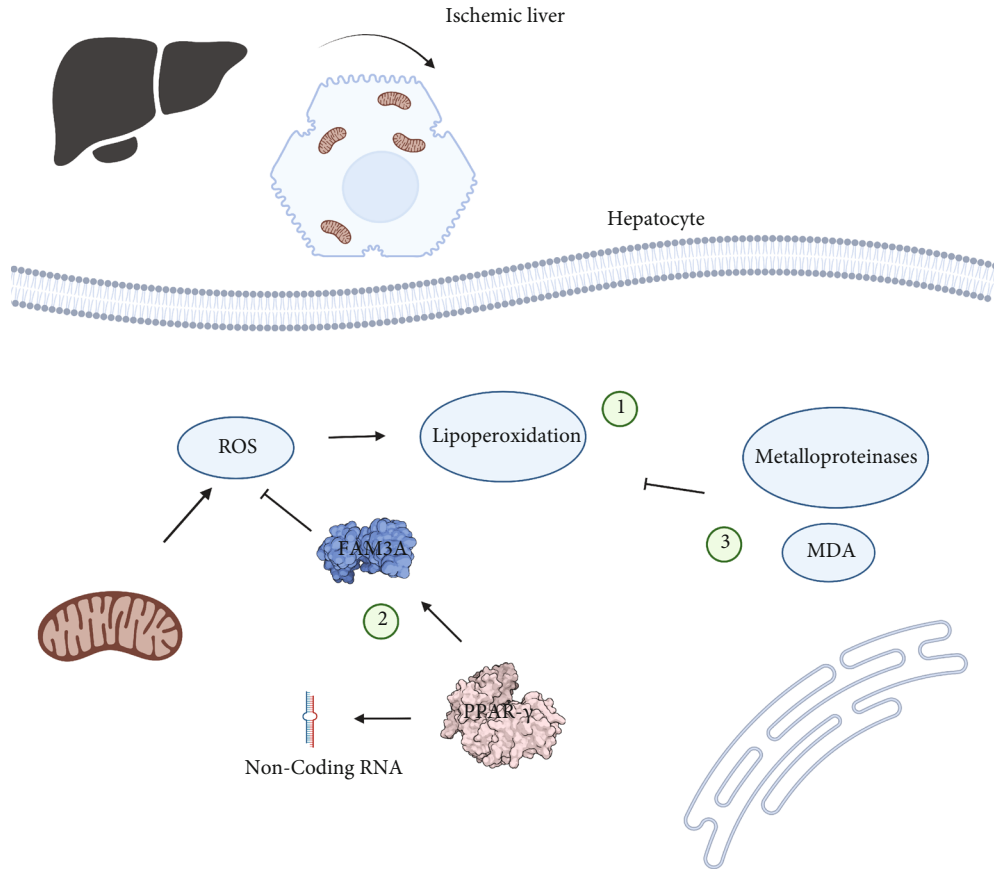

Figure 3: Main targets to regulate oxidative damage during hepatic ischemia. There are three principal points in hepatic tissue when oxidative damage is modulated. (1) Lipoperoxidation interferes with normal cellular functions and is the principal objective during hepatic I-R. That is why the peroxisome proliferator-activated receptor-gamma (PPAR-γ) is considered a good target. (2) It inhibits ROS production via the FAM3A complex and noncoding RNA. Besides, ROS formation of release blockade is crucial for diminishing lipoperoxidation, in addition, with the activity of metalloproteinases and malondialdehyde antioxidative complex.

damage [116, 117]. Therefore, some interesting targets are the SOD, CAT, and GPX receptors, since, without a doubt, the neutralization of ROS and hydroperoxides mediated by these enzymes is a probable target for the treatment of renal I-R [118]. Notably, although antioxidant therapy is a viable alternative, damage caused by I-R cannot be fully treated. However, its efficiency is sufficient to considerably reduce oxidative damage [62, 118]. Some studies have shown the beneficial effects of free radical scavenging molecules on renal I-R. Molecules including melatonin can modulate the damage induced by renal reperfusion in ischemic kidneys due to its antioxidant activity [119]. Moreover, lipoperoxidation is a proven mechanism with good outcomes in renal tissues, where we could highlight the increase in SOD activity as the main target [120]. Furthermore, Diao et al. showed that the inhibition of protein arginine methylation transferase 5 (PRMT5) blocked ROS-mediated pyroptosis via the Nrf2/HO-1 signaling pathway. Therefore, PRMT5 is an interesting management target in renal I-R injury [121, 122]. The mitochondrial receptor MnSOD, an antioxidant enzyme capable of scavenging O_2 free radicals, while controlling peroxynitrite radical (ONOO-), can successfully modulate I-R in renal tissues [123]. Although the role of ferroptosis in the renal I-R phenomenon has not been completely elucidated, several molecules can be targeted and provided interesting possibilities for therapeutics [124]. Pannexin 1 is an ATP-releasing protein that exhibits proapoptotic properties in renal I-R [125]. With consideration of molecular targets for modulating ferroptosis, the GPX4 enzyme can be a key regulator of lipoperoxidation [126]. Therefore, its activation is strictly correlated with the process of cell death and, consequently, the accumulation of ROS. This mechanism was found to be successful in pharmacological alternatives including irisin [127] (Figure 4).

7.4. Brain Tissue. The brain is the most sensitive organ to blood supply interruption without the possibility of repair in I-R. That is, 20 minutes of ischemia is enough to exceed the threshold of damage it can withhold [18]. That can potentially cause or lead to oxidative stress-induced behavioral and cognitive decline. Oxidative stress in the brain caused by I-R leads to the primary etiologies of brain damage and significant neuronal effects, resulting in tissue destruction and cell death. These include lipid peroxidation, protein denaturation, inactivation of enzymes, nucleic acid, and DNA damage, the release of Ca^{2+} from intracellular stores, damage to the cytoskeletal structure, and chemotaxis [128]. Phospholipids in the brain are vulnerable to ROS-

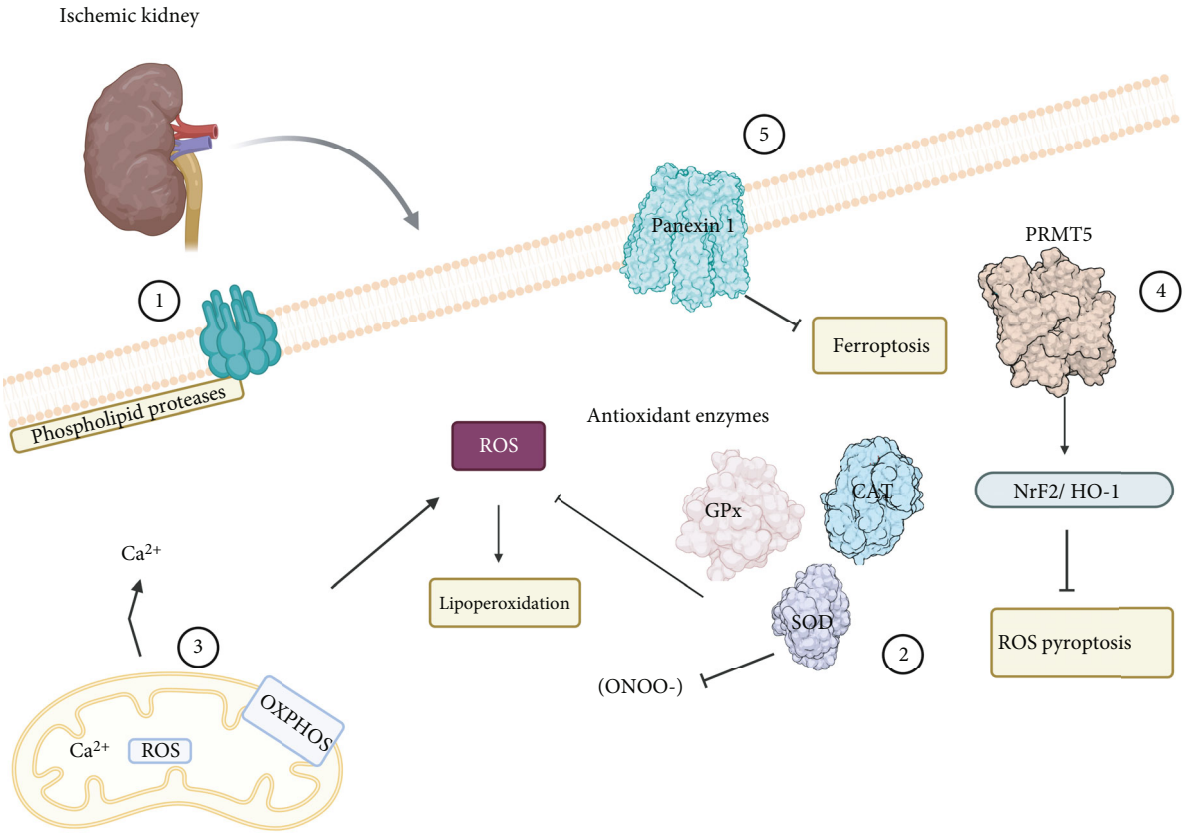

Figure 4: Relevant targets during ischemia-reperfusion in renal tissue. The main ways to decrease renal oxidative damage during I-R are (1) activation of phospholipid proteases, (2) the increment of antioxidant enzyme complex reducing free radicals and blocking lipoperoxidation, (3) modulation of Ca^{2+} and ROS mitochondrial release as well as controlling oxidative phosphorylation, (4) PRMT5 protein arginine methylation transferase is a regulation mechanism to stop ferroptosis, and (5) the membrane receptor Pannexin 1 through that blockaded ROS pyroptosis through Nrf2/HO-1 signaling pathway.

mediated peroxidation. However, proteins and DNA are targeted by ROS, and they become problematic with aging as aging brains exhibit high oxidative stress-induced mutation levels in the mitochondrial DNA [129, 130].

Perhaps, we cannot find an effective therapeutic target for reducing this damage. That is why ischemic preconditioning combined with antioxidant therapies will most likely be critical regulators for ischemic stroke [131]. Current studies have focused on the pathways of oxidative stress that involve a variety of cellular pathways, receptors, and processes that can be used on focused therapy for oxidative damage, such as autophagy, mitophagy, and necrosis, which are involved in eliminating excess ROS and subsequent cell death triggered by these free radicals [132, 133]. The endogenous protective mechanisms in the brain included the antioxidant enzyme systems and the low-molecular-weight antioxidants [134]. In response to stress, cells increase their antioxidant defenses with nuclear factor erythroid 2-related factor (Nrf2), an important transcription factor [135]. Therefore, Nrf2 has been proposed as a pharmacological target in pathologies with oxidative features since it modulates several genes encoding antioxidants and detoxification enzymes such as heme oxygenase 1 (HO-1), NAD(P)H dehydrogenase quinone 1, superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1), and catalase (CAT) [136]. By contrast, mitochondrial dysfunction suggests several diseases, including neurodegeneration [137].

The mitochondrial role in ischemic shock and its pathogenesis mainly involves the formation of free radicals [138]. mtDNA is particularly susceptible to oxidative damage because of its proximity to high levels of mitochondrial ROS production and its relatively poor defense against damage. Healthy mitochondria contribute to oxidative stress resistance by increasing respiratory capacity [139]

Taken together, ATP synthase and the electron transport chain make up the OxPhos system, which is the leading promoter of the mitochondrial electrochemical gradient [140, 141]. Crucial key points for electrons to enter are complex I and II of the electron transport chain, which through ubiquinone transfer electrons to complex III and this in turn to complex IV. Proton pumping via the mitochondrial membrane is the primary mechanism for maintaining the membrane potential. These protons are then used by complex V ATP synthase to form ATP and complete the oxidative phosphorylation process [142]. Reversible phosphorylation mechanisms are relevant targets of this whole process. Preserving these phosphorylation epitopes could offer a regulatory control for reducing oxidative damage since it could

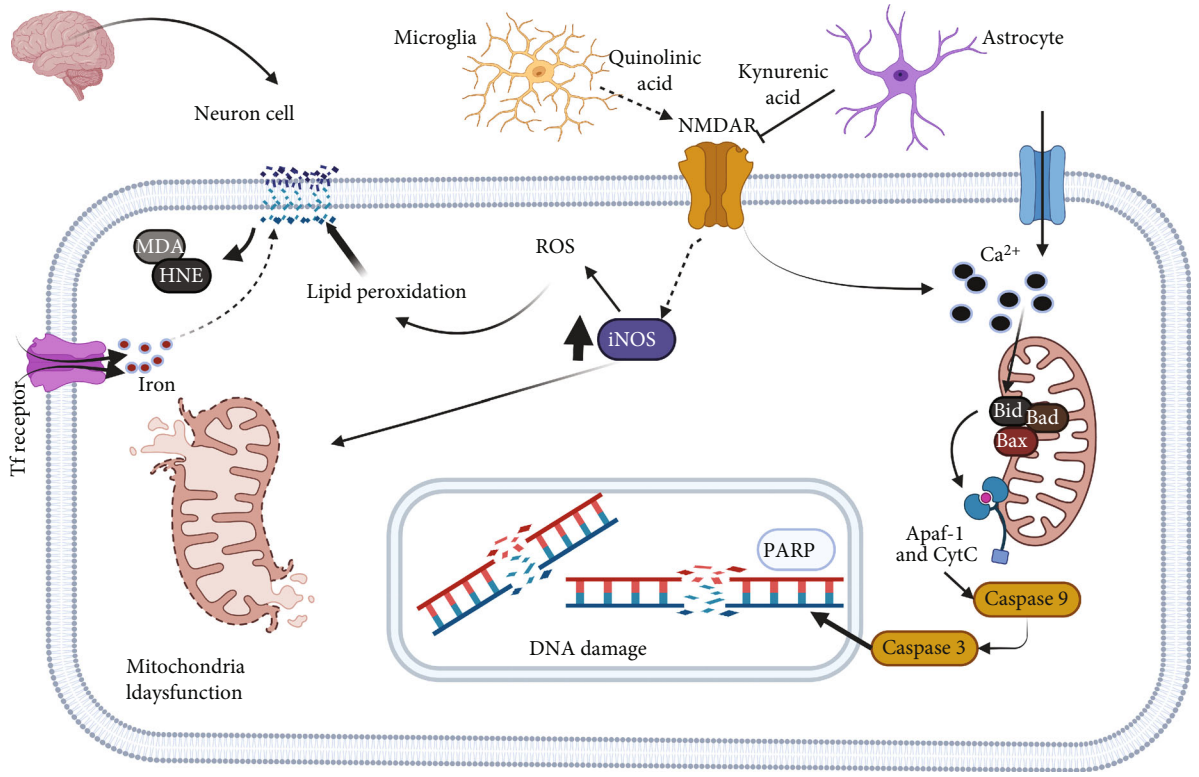

Figure 5: Oxidative stress in neuron cells. Several mechanisms activate oxidative stress during ischemia in neuron cells, principally leading by the quinolinic acid released by microglia and mitochondrial Ca^{2+} overload and ROS formation after BLD, BAD, BAX complex, and APAF-1 and CytC activate caspases pathways that result in DNA damage. Nevertheless, astrocytes intend to block that oxidative stress and lipoperoxidation that could damage plasmatic membrane releases kynurenic acid. Unfortunately, Tf receptors promote cell damage through ferroptosis. Therefore, all of these would function as excellent regulatory points for I-R oxidative injury in nervous tissue.

allow the regulation of OxPhos mediated by calcium and the ADP-shuttle mechanism [143]. The OxPhos complexes are phosphorylated in vivo by the second messenger Ca^{2+}, thereby triggering the phosphorylation of most mitochondrial proteins, a process mediated by calcium-dependent phosphatases during ischemic stress [144]. This phosphorylation alters the electron transfer kinetics, which affects the allosteric regulation of ATP and ADP [145]. The mitochondrial membrane potential ($\Delta\Psi m$) becomes positive in the inner chamber during oxidation, and cytochrome c (CytC) is released into the cytoplasm. The release of CytC from the mitochondria is an important pathway for the cascades of apoptotic events [146]. These proapoptotic proteins, such as Bid, Bad, Bax, Bak, Bok, and Bim, in the outer mitochondrial membrane, increase the permeability of membranes, thereby forming specific pores and stimulating free CytC release. Third, CytC binds to apoptosis protein-associated factor 1 (Apaf-1) and forms the Apaf-1/caspase-9/CytC complex. Finally, caspase-3 is activated, which triggers apoptosis and delays neuronal death [147]. Lipid peroxidation is one of the significant consequences of ROS-mediated injury to the brain. This ultimately leads to the production of conjugated diene hydroperoxides that attack lipids containing carbon-carbon double bond(s) in specific polyunsaturated fatty acids (PUFAs). Among these compounds, malondialdehyde (MDA) and HNE are the breakdown products of lipid peroxidation, and they are elevated in patients with ischemic stroke [148], with infarct size, stroke severity, and patient outcome. MDA can be the most mutagenic lipid peroxidation product, and HNE is the most toxic [149]. MDA is widely used as a biomarker for lipid peroxidation of omega-fatty acids, HNE is a cytotoxic product originating from peroxidation, and it is considered as one of the significant toxic products generated from lipid peroxides. The highly toxic characteristic of HNE can be explained by its rapid reactions with thiols and amino groups [150]. HNE is a bioactive marker of lipid peroxidation and is a signaling molecule involved in the regulation of several transcription factors, such as nuclear factor erythroid 2-related factor 2 (Nrf2), activating protein-1 (AP-1), NF-κB, and peroxisome proliferator-activated receptors (PPAR), cell proliferation and differentiation, cell survival, autophagy, senescence, apoptosis, and necrosis [151]. Hemoglobin (Hb)/haem is a putative neurotoxin. Hb is the most abundant protein in the blood and is released from lysed red blood cells after stroke. It can be engulfed by the microglia in the perihematomal zone and metabolized into ferrous/ferric iron, which induces ROS formation and lipid peroxidation [152]. The excess ferrous iron accumulates in the neurons via the transferrin (Tf)–Tf receptor system that forms highly toxic hydroxyl radicals ($^{\bullet}OH$). These hydroxyl radicals attack DNA, proteins, and lipid membranes, leading to the disruption of cellular function. Ferroptosis was found in organotypic hippocampal slice cultures exposed to

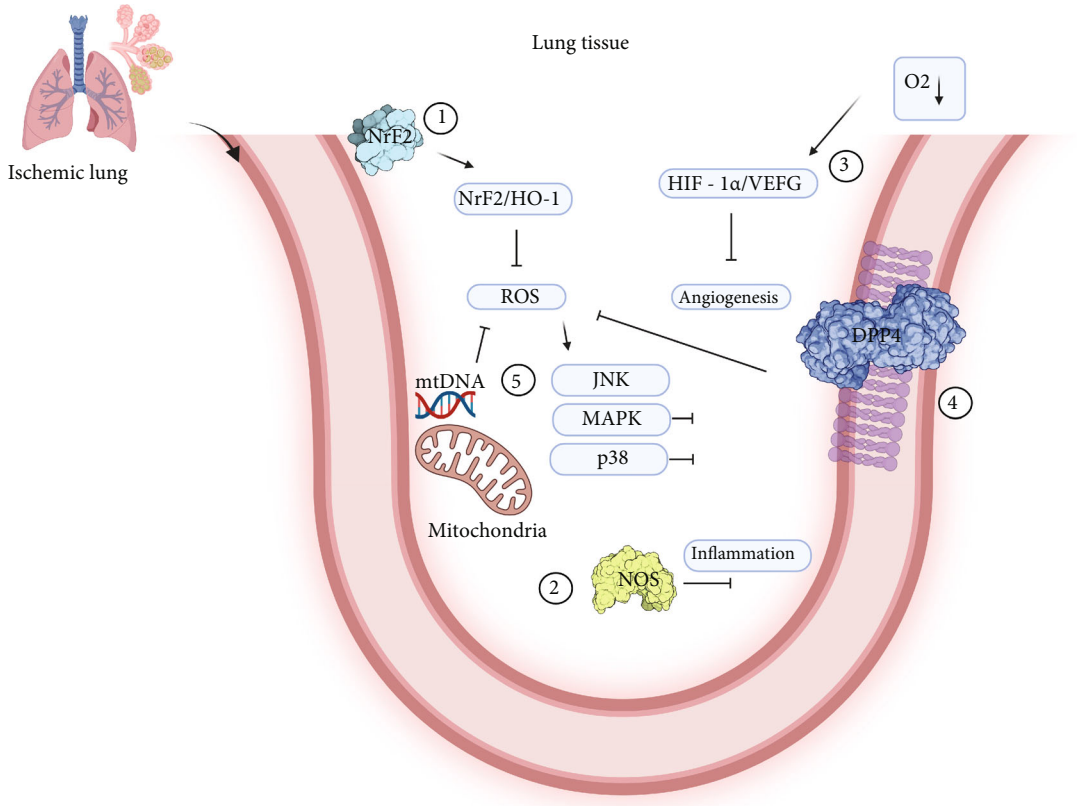

Figure 6: Molecular targets to decrease oxidative damage during I-R. (1) One of the main points to reduce oxidative damage is the NrF2 factor, the starting point of the NrF2/HO-1 signaling pathway that triggers ROS blockade. (2) Nitric oxide synthase, being blocked, can prevent the activation of the inflammatory cascade. (3) Low oxygen concentrations in the cell lead to the activation of HIF1 and HIF 1α/VEGF, one of the primary mechanisms for generating new vasculature. (4) DPP4s decrease oxidative stress when their expression increases. (5) In addition, mitochondrial DNA can function as a signaling mechanism that allows the activation of programs to block the production of reactive oxygen species.

glutamate [153]. It can be distinguished from other types of regulated cell death because it does not require caspases ATP depletion or mitochondrial ROS generation (Bax/Bak) or elevations in intracellular Ca^{2+} levels [154]. Ferroptosis is triggered by glutathione biosynthesis or glutathione peroxidase 4 (GPX4) activity inhibition and is associated with shrunken and electron-dense mitochondria morphologically [155]. Tryptophan (TRP) is an aromatic essential amino acid whose route of TRP metabolism is the kynurenine (KYN) pathway, and the primary end products are nicotinic acid and its derivatives and NAD^+ and NADP, which are two ubiquitous coenzymes In this catabolic process, starting from the central compound, kynurenine (KYN) forms kynurenic acid (KYNA), xanthurenic acid (XA), and picolinic acid [156] (Figure 5). KYNA is produced mainly in astrocytes, and quinolinic acid (QUIN) degradation occurs in microglial cells in the central nervous system. More recently, tryptophan oxidation via the kynurenine pathway has been implicated in inflammation and oxidative stress in the brain that occurs after stroke [157]. Elevated QUIN levels can cause excitotoxic cell death. The hippocampus and striatum are most sensitive to QUIN neurotoxicity. QUIN can directly interact with free iron ions to form toxic complexes that exacerbate ROS formation, oxidative stress, and excitotoxicity [158]. Moreover, it induces lipid peroxidation, produces ROS increases iNOS expression, decreases SOD activity, and causes mitochondrial dysfunction QUIN which stimulates mitochondrial dysfunction and apoptosis [159]. By contrast, the advantage of KYNA is that it cannot be metabolized to excitotoxic agents and scavenges oxygen radicals, thereby decreasing cellular damage. The application of KYNA in high concentrations or for a prolonged time causes neuronal cell damage [160]. The multiple effects of the kynurenine pathway and its changes during stroke have increased in recent years, thereby allowing interference with therapeutic targets. DNA damage includes oxidative modification and endonuclease-mediated DNA fragmentation. DNA oxidation may activate repair enzymes, such as poly (ADP-ribose) polymerase (PARP). PARP activation progresses from neuronal elements and localization of infiltrating inflammatory cells 3–4 days after stroke. The activation of PARP leads to DNA injury in the brain [161]. Indeed, there is a strong association between oxidative stress and PARP activation in the brain, and oxidative stress in the neurons can induce PARP activation [162]. PAR can directly affect mitochondrial membrane potential collapse [163].

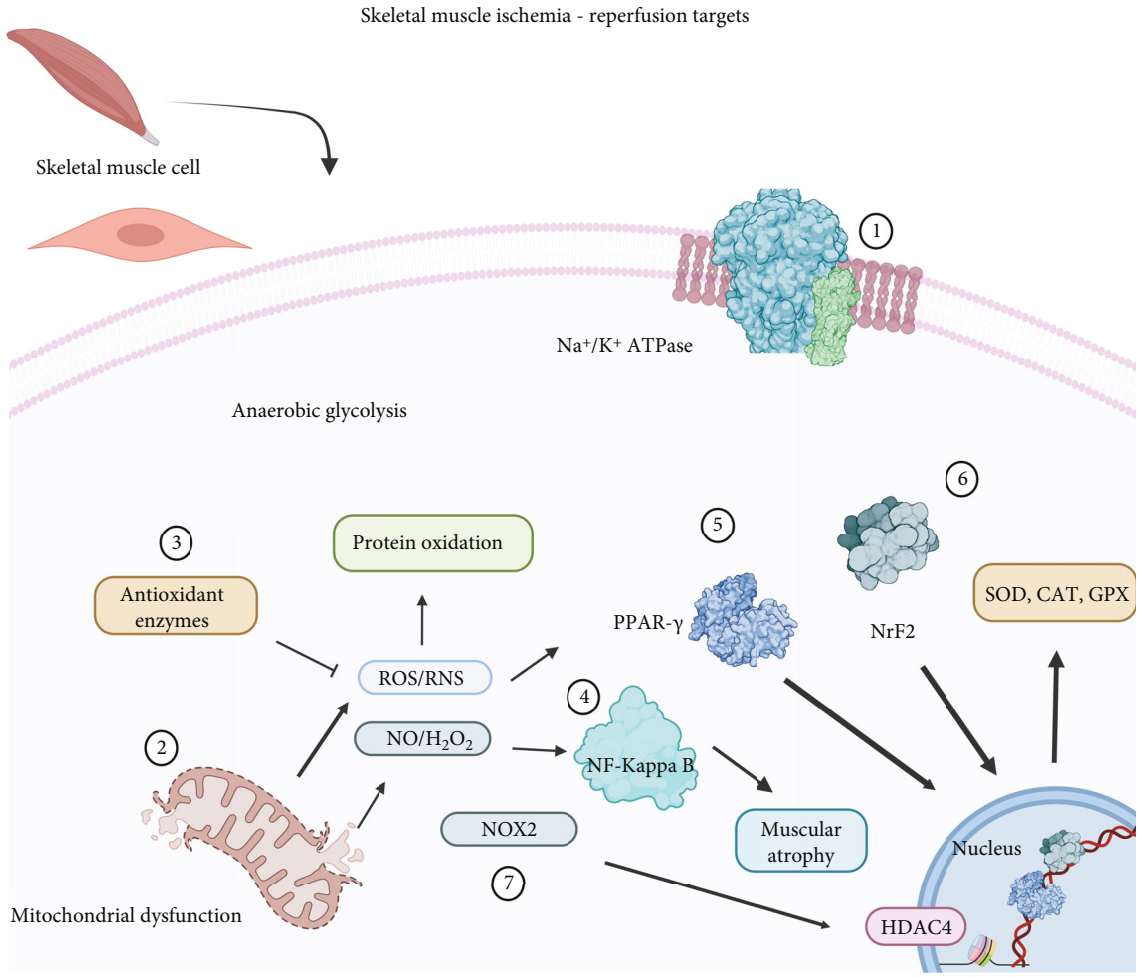

Figure 7: Skeletal muscle I-R targets. (1) Na+/K+ ATPase activation promotes the saturation on Na+ and K+. That is why modulation of these enzymes could be an excellent alternative to reduce damage. (2) Blocking mitochondrial release of ROS and RNS during dysfunction could improve cellular damage. (3) Scavenger activity of antioxidant enzymes is one of the main alternatives to modulate this condition. (4) Activation NF-κB leads to muscular atrophy for several inflammatory mechanisms. (5) PPAR-γ translocations, as well as NrF2 (6), result in the expression of the antioxidant complex. (7) NOX2-depending pathways conduce to activation of HDAC4 that facilitates gene expression.

Thus, PARP-1 activation may inhibit glycolysis and cause energy depletion, thereby leading to altered cellular metabolism.

7.5. Lung Tissue. The lungs are affected by I-R indirectly. Several signaling pathways such as Nrf2/HO-1 and HIF 1α/VEGF have protective effects on this organ [164]. NrF2 is a transcriptional factor that protects cells from stress, and oxidative processes activate NrF2 to initiate such an effect. In turn, HO-1 becomes a rate-limiting enzyme that can reduce oxidative stress by increasing its expression in the lung either via local or peripheral ischemia [165, 166]. Hypoxia-inducible factor 1-α (HIF-1α) and cell repair mechanisms mediated by VEGF are implicated in the regulation of angiogenesis in ischemic events [167, 168]. After ischemia damage to the lungs, there is a significant loss of plasma proteins and inflammatory cells, and there are high amounts of HIF-α and its regulatory target VEGF during I-R in local tissues [169]. Moreover, recently, the close association between these two molecules has been correlated with repair mechanisms independent of angiogenic activity [164]. The mitochondrial approach may also be a good alternative to modulating oxidative damage. Some reports have shown interesting results regarding protecting the integrity of the mitochondrial DNA using the oxidative approach. That is, mtDNA could serve as a sentinel of ROS-mediated functions, as observed primarily in the lung tissues [170]. Using conventional oxidative stress as a therapeutic target in ischemia could be complicated. Therefore, preconditioning alternatives can be another therapeutic option. Researchers have designed in vitro and ex vivo experimental data in which the tissues and cells are exposed to high concentrations of polyethylene glycol-catalase (PEG-CAT) to protect against cytotoxicity caused by oxidative stress. This mechanism then preserves cellular metabolism and mitigates pulmonary I-R. Therefore, PEG-CAT can be an important therapeutic target [171]. Anti-inflammatory approaches for decreasing pulmonary ischemia remain unclear. It was proposed that the establishment of novel therapeutic strategies should involve the inhibition of transcriptional factors that activate oxidative stress with better techniques. For example, MAPKs that are activated after oxidative stress in the inflammatory

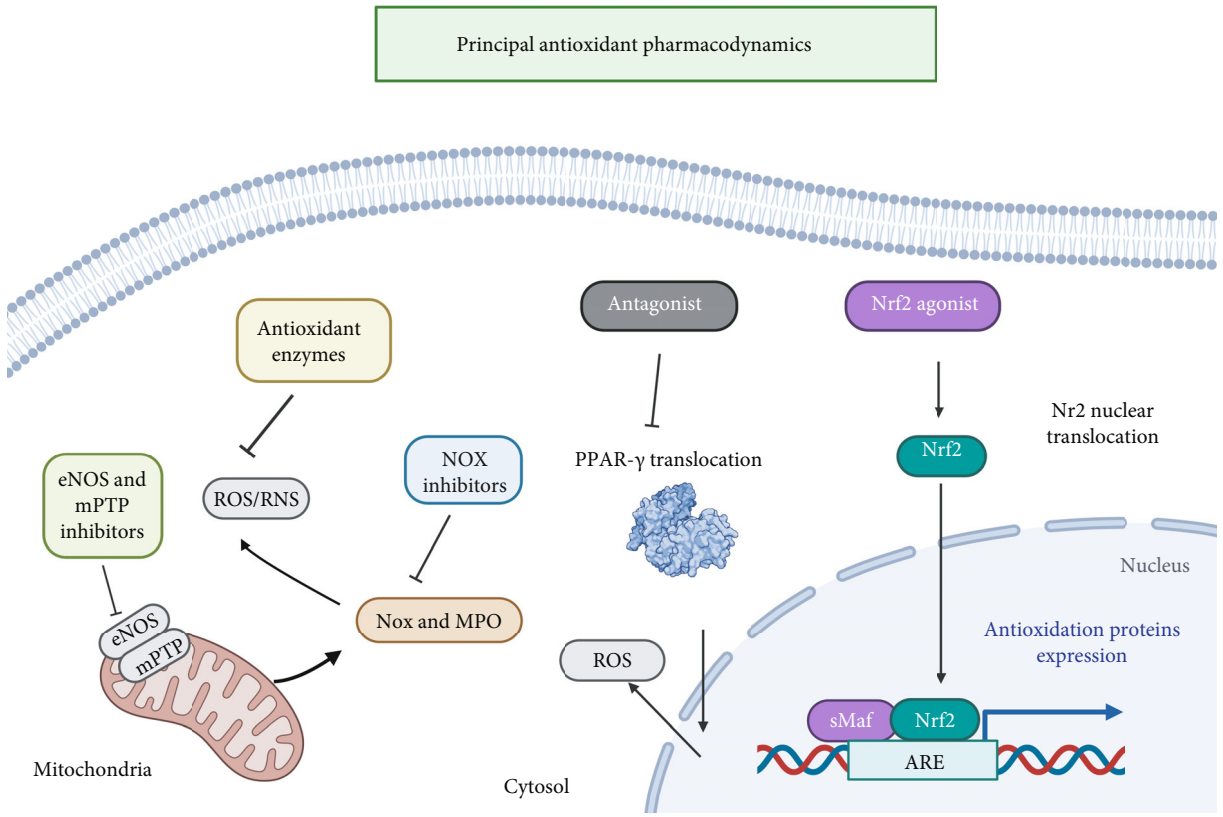

Figure 8: Principal antioxidant pharmacodynamics. The enlisted mechanisms are promising targets that are part of the pharmacodynamics of several drugs used in clinical practice. Antioxidant enzymes as a scavenger to reduce ROS and RNS, NOX inhibitors blocking free radical production, eNOS and MTTP inhibitors leading to reduction of reactive species and avoiding is release. Nuclear factors agonism and antagonism regulate the antioxidant expression and ROS production.

models of pulmonary ischemia and different signaling pathways, such as p38, c-jun N-terminal kinase, p38 inhibition, or JNK, have protective effects in this organ [172]. In addition, ROS and RNS mediate inflammatory reactions by activating alveolar macrophages. With the activation of the inflammatory cascade, multiple potential ROS generators such as the mitochondria, xanthine oxidase, NOX, NOS uncoupling, and neutrophils must be considered as a therapeutic target for oxidative damage [173]. By contrast, the expression of DPP4 is directly correlated with decreased oxidative damage. That is, the capillaries are the main concentration regions of this expression, and DPP4, a serine protease, commonly cleaves the substrates with proline and alanine in the latter position [174] (Figure 6).

7.6. Skeletal Muscle. In the limbs, skeletal muscle is the predominant tissue, and pathophysiological literature indicates that the damage threshold of this tissue is exceeded after 3 h of ischemia and is irreversible at 6 h [175]. Some studies about I-R showed that the main mechanisms of cell damage and death are mitochondrial dysfunction and mitochondrial proapoptotic protein release [176]. Similar to other cells, I-R in the myocyte is mediated by the mitochondrial membrane potential and the proton gradient that promotes ATP synthesis via oxidative phosphorylation [177]. This reduction during the ischemic process promotes ATP synthesis and inhibits Na^+/K^+ ATPase, thereby increasing intracellular Na^+ and Ca^{2+} and anaerobic glycolysis. Further, the mitochondria play an important role in the pathophysiology of I-R in this tissue, and the free radicals generated by the skeletal muscle during rest and activity are NO and superoxide, which is dismuted into H_2O_2. However, there are still several limitations, and few studies have identified the nature of ROS or RNS present in the muscle fibers. Most reports have only examined cell surface free radicals [152]. Consequently, there are only a few reports about NO or H_2O_2 or substances that can cross mitochondrial barriers, but there are a large number of reactive species that have not been confirmed to be involved in skeletal muscle physiopathology [152]. To avoid deleterious effects on tissues, there are several cellular mechanisms to modulate free radicals such as the mitochondrial and cytosolic isoforms of superoxide dismutase (MnSOD and CuZnSOD) in addition to CAT and GPX modulation of their expression [178]. Any cellular processes are regulated by ROS and RNS, such as the activity of transcriptional factors, ionic transportation, apoptosis, and metabolism [179]. Proteins in skeletal muscle are susceptible to oxidation of their sulfhydryl groups or the formation of

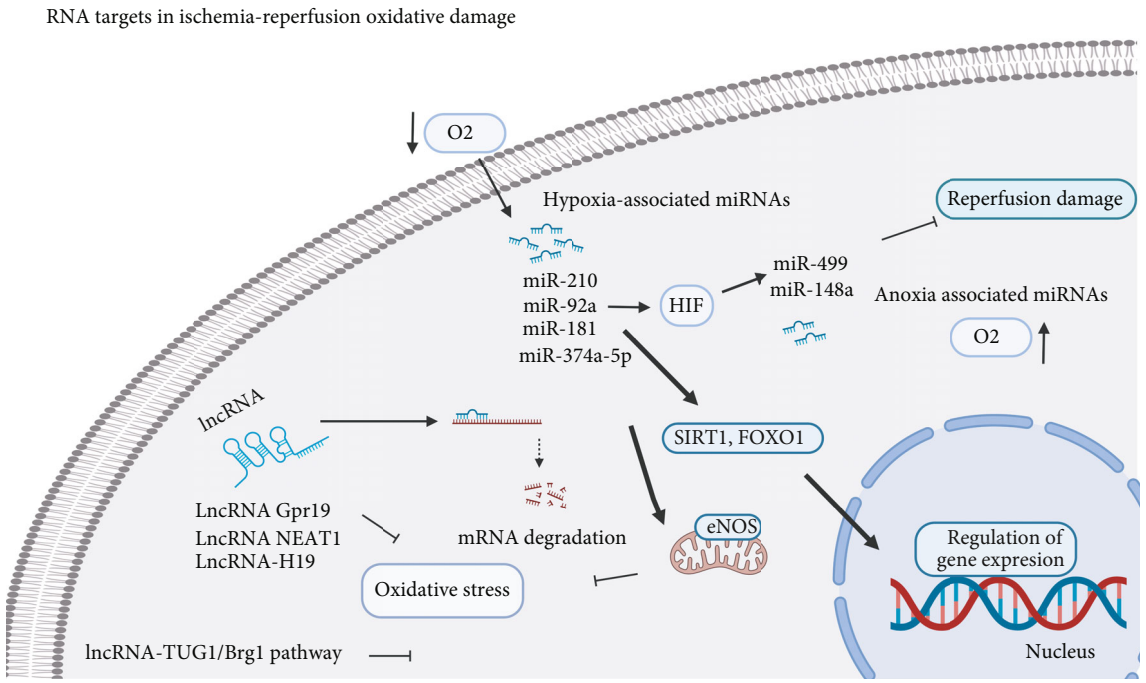

Figure 9: RNA targets in ischemia-reperfusion oxidative damage. We can make a group of the different miRNAs among those directly associated with the decrease in oxygen or hypoxia that directly activate the expression of HIF or are sensitive to it. Noncoding RNA activates various transcription factors to regulate the gene expression of antioxidant enzymes and prevent the formation of reactive species that exacerbate oxidative stress. Furthermore, those related to reperfusion or anoxia mechanisms are responsible for the resolution or inflammatory mechanism. Similarly, various lncRNAs block signaling pathways to reduce oxidative stress.

disulfide bonds. These processes are involved in the modulation of protein functions [180]. In the same way, ROS also functions as excellent second messengers in the activation of apoptosis programs such as the NF-kappaB pathway, which is involved in muscle degeneration and atrophy [181]. Several alternatives of regulation, such as the PAR-gamma coactivator-1 alpha (PGC1-α) pathway, are redox-sensitive, in which ROS would play a regulatory role [182]. In addition, ROS dependent on Nox2 is involved in the regulation of histones such as histone deacetylase 4 (HDAC4), this happens during vigorous muscle activity, as a regulation mechanism [183]. Not less important is the already described NRF2 transcriptional factor involved in multiple regulations of antioxidant defense [184]. Under conditions of oxidative stress, NRF2 is found in the cytoplasm thanks to the activity of degrading proteins; after it is released, it translocates into the nucleus where it activates the transcription of antioxidant gene programs and their respective protein [185]. Although it well tolerates oxidative damage, these modulation strategies are essential for the resolution of countless pathologies correlated with ischemia [186] (Figure 7)

8. Ischemia-Reperfusion Antioxidant Pharmacodynamics

Antioxidant therapy has been used to modulate oxidative stress in different experimental models. Generally, some proven strategies are used as antioxidant preconditioning without completely effective outcomes [7, 187, 188]. This can be explained by the nonselective characteristic of ROS modulation, which directly interferes with cell signaling pathways [189]. This has led to alternative approaches such as activation of the Nrf2 pathway by fumaric acid derivatives, resulting in a proven antioxidant activity [190]. Another relevant strategy is using ROS-producing enzymes such as Nox and MPO, which induce a more specific response by modulating pathological conditions [191, 192]. However, the most promising approach involves enzyme activity, particularly via drugs with a potential to reverse eNOS activity in pathologies correlated with oxidative stress [193]. However, despite advancements, almost all innovative cardiovascular therapies have been inadequate in the management of these pathologies.

8.1. Free Radical Scavengers. Notably, reactive species at low concentrations fulfill cellular functions as metabolic bioproducts or second messengers. At high concentration, they have deleterious effects, mainly in pathological events such I-R [194]. These effects conceptualize as oxidative stress and lead to the opening of mPTP, resulting in protein and DNA damage [195]. ROS signaling can be interfered with via the inhibition of complex I, using drugs such as metformin. This then reduces the amount of ROS in the cytosol [196].

8.2. Mitochondrial Respiration Chain Blockers. The electron transport chain stands out during the reperfusion process. This explains why direct modulation can be an alternative for reducing ROS production and the consequent

Table 1: Pharmacological approaches against I-R pathologies.

Family	Pharmacodynamic	Results
β-Blockers	Reduce the cardiac frequency and calcium overload blockade	Regulate myocardial infarction
Glucose modulators	Regulate glucose/insulin/potassium concentration	Reduce myocardial infraction and infarct size
Immunomodulators (abciximab)	Reduce Inflammation and oxidative stress activation	Reduce infarct size in acute coronary syndrome
Inhaled NO and $NaNO_2$	Regulates oxidative stress	Failure in reducing myocardial infarction
MPTP inhibitors	Blockade of mitochondrial ROS release	Adverse effects and not significant data
Statins	Oxidative scavengers and IL10 expression	No significant data in acute coronary infarction
ARA II	PPAR-γ expression and antioxidant activity, SOD2 expression	Significant data were preventing I-R

activation of mPTP with cell death as an outcome [197]. The deregulated production of ROS in the mitochondrial respiratory complexes is associated with the I-R process. Regulating these processes modifies the harmful nature of ROS to a protective one, and these respiratory complexes are the main targets to carry this out [198]. Complexes I and III are major superoxide production sites. Electrons are transferred along the chain and back to complex I where NAD+/NADH is reversed and ROS production increases [199]. Using this approach, several drugs have been tested under I-R conditions, thereby providing varying but important results for understanding the phenomenon [200–203]. Some reports used the reversible inhibition of transiently inactivated complex I to diminish the generation of ROS without losing its function [59, 204, 205]. In these categories, some compounds such as biguanides, amobarbital, nicorandil, rotenone, and S-nitroso-2-mercaptopropionyl glycine have shown interesting results [205–210]. Highlighting metformin has exhibited cardioprotective properties by modulating complex I at high doses [211, 212]. Some strategies use acidic citric intermediates, malate, and oxaloacetate to inhibit complex II. Although it is not a specific site for the formation of ROS during the reperfusion process, it is correlated with complex I and III modulations, which allows cardioprotection regardless of K^+ concentrations [213, 214]. Notably, some important mechanisms are not directly involved in the production of ROS nor as second messengers in the adaptation mechanism to hypoxia [215]. These signaling pathways are directly linked to ischemic preconditioning in different pathological conditions [216]. A consecutive modulation of complex III via the ubiquinol oxidation center (Qo site) has shown cardioprotection [217]. Reduced cytochrome c activity has a similar effect in electron transport from complex III to IV, an event that reduces superoxide-free radical production, which is a poorly understood mechanism [218] (Figure 8).

8.3. MPTP Inhibitors. The importance of MPTP for the development of I-R-mediated oxidative damage has been discussed. They represent a key point for the release of ROS/RNS from the mitochondria [36]. However, the therapeutic target approach of this protein complex is via its subunits, with CyD as one of the main ones, to which countless drug prototypes have been designed [219]. Nevertheless, in vivo and clinical data are not favorable enough to establish a therapy [220, 221]. Some analog drugs of CyA have shown favorable outcomes in myocardial, hepatic, and cerebral animal models [222]. Even some drugs that are not CyA analogs have interesting outcomes via this pathway in vivo models [223]. Other than this therapeutic target, other possible alternative therapies such as N-phenylbenzamides and cinnamic anilides can inhibit mPTP activity, thereby providing protective effects against oxidative damage [224, 225]. Nrf2 and NF-κB regulators are also good alternatives to ameliorate oxidative damage via this pathway [226–228] (Figure 1).

8.4. PPR's Gamma Inhibitors. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is the target of multiple studies about cardiovascular pathologies [229]. Several isoforms have been described. However, the γ isotype is the most relevant in these I-R-related diseases [230]. After binding to endogenous ligands, the retinal X receptor is heterodimerized with a nuclear receptor, thereby inducing or repressing gene expression [231]. Therefore, PPAR has relevant roles in hepatic IR injury [232]. Some angiotensin II drugs are associated with this receptor, which exhibits an inhibitory effect [233, 234]. Several drugs attenuate PPAR I-R via antagonism, thereby reducing ROS production.

8.5. RNAS Transcripts in I-R. RNA and DNA are targets for modulating gene expression. Currently, advancements in molecular biology can allow them to be used as excellent therapeutic targets in multiple pathologies [235]. For example, the modulation of antioxidant enzymes using gene therapy has been useful in oxidative stress if a specific target receptor is already known [236]. Redox homeostasis has a direct correlation with cell function. Redox imbalance leads to oxidative stress production, which inhibits the development of vascular diseases and I-R injury, as well as triggers transcriptional and posttranscriptional modulation in gene expression [237, 238]. In addition, hypoxia is considered an important stimulus to regulate microRNA (miRNAs) expression [239]. Some miRNAs, known as hypoxia MIR, are even associated with

hypoxia, and some of these transcripts are involved in the pathophysiology of ischemic and cardiovascular diseases [240]. An example is miR-210, a hypoxia-inducible transcript that promotes cell survival and improves cardiac function via antiapoptotic and angiogenic mechanisms [241]. Moreover, HIF is regulated by these transcripts with an important role in these pathologies [242]. Other examples of these nuclei of response to oxidative damage are NRF2, FOXO1, and NF-kB in the inflammatory part [243–247]. And the widely studied transcriptional factor p53, among the multiple stimuli that activate it, is ROS one of them, which consecutively triggers proapoptotic and antiproliferative mechanisms [248]. Even a single oxidative stress stimulus, such as H_2O_2 and O_2, could be correlated with complex redox imbalance mechanisms in pathologies such as aging and limb ischemia [249, 250]. However, even though the role in oxidative stress of noncoding transcripts (miRNA and lncRNA) is widely known, their use as a therapeutic strategy remains premature [251, 252]. miRNAs are RNAs that regulate gene expression by forming hybrids with mRNAs, altering their translation [253]. Moreover, they have a regulatory role in oxidative stress via their interactions with SIRT1, FOXO1, and eNOS [254]. Furthermore, in different pathologies correlated with oxidative stress, these regulatory mechanisms have begun to take center stage [224] In I-R, miRNAs have great advantages when used as a therapeutic target. In the future, they could be considered pharmacological approaches in clinical practice [255]. Noncoding RNA similar to miR-92a has a proangiogenic effect, and miR-499 can decrease damage induced by hypoxia-reoxygenation. miR-24 had a similar mechanism correlated to the attenuation of infarct size in animal models [256–258]. Recently, the use of novel noncoding RNAs as therapeutic alternatives is emerging. For example, miR-181 is a drug target with an effect on experimental myocardial I-R [259]. miR-148a alleviates hepatic I-R and is implicated in resolution pathways [260, 261]. Another alternative is miR-374a-5p. This decreased myocardial cell damage in an I-R model [262]. Small RNAs are not the only noncoding transcripts relevant to disease-related I-R. Long RNAs (lncRNAs) are expressed by an opposite strand of mRNA, and they are located in the introns of annotated genes and transcribed from enhancer regulatory elements (eRNA) [263]. Moreover, this transcript mechanism varies, ranging from repressors to activators of gene expression, or even posttranductional regulators. They are also mRNA splicing and stability modulators; lncRNAs play an important role in regulating the response to oxidative stress [264]. For example, lncRNA Gpr19 inhibits and attenuates (I-R) injury after acute myocardial infarction, and lncRNA NEAT1 alleviates sepsis-induced myocardial injury by modulating oxidative stress [265, 266]. Some drugs including propofol have shown a beneficial effect against I-R oxidative stress under different conditions via the lncRNA-TUG1/Brg1 pathway in liver cells in an IR model [267]. Drugs such as metformin regulate oxidative stress via transcripts such as lncRNA-H19 [268]. In a recent study, ZFAS 1 lncRNA was found to reduce ischemic stroke via the regulation of some oxidative stress mechanisms [269]. (Figure 9).

9. Alternative Interventions in Clinical Trials

Regardless of the several researches carried out to decrease damage caused by I-R injury, there is still no relevant pharmacological therapy that reduces damage caused by this condition in clinical practice. However, some studies have shown important data, particularly about those regarding Cyst A, in advanced pharmacological phases or pilot studies [270]. By contrast, metoprolol, a β-blocker, exhibited interesting activity in patients with I-R induced by myocardial infarction [271, 272]. Moreover, some glucose modulators have inhibited I-R damage via several mechanisms, with glucose/insulin/potassium (GIK) as the primary on [273]. In terms of clinical results, patients with acute coronary syndrome have presented with a significant reduction in infarct size [274]. In addition, intracoronary administration combined with thrombectomy significantly reduced infarct size [275]. The application of novel therapies in clinical practice is challenging, and there is an extensive list of not favorable outcomes in I-R treatments in clinical trials. One of the main reasons for these failures is the lack of clarity and significance in preclinical trials, thereby making it impossible to obtain relevant clinical information [276]. The most relevant examples are studies about inhaled nitric oxide and sodium nitrite for myocardial infarction. These strategies were tested in clinical practice. However, they were not effective in reducing infarct size [277]. The use of TRO40303, an MPTP inhibitor, had unfavorable outcomes in the MITOCARE study [253]. In the TREAT study, the outcomes of ticagrelor and clopidogrel treatment in similar pathological conditions were not significant [278]. However, some reports highlight interesting epitopes or therapeutic approaches, thereby allowing the appropriate incorporation of new clinical evidence into the practice guidelines of phase III trials, which are required to assure a solid preclinical background (Table 1).

10. Perspective

The therapeutic targets most likely to be extrapolated in clinics settings are not yet fully elucidated. Hence, further studies should be performed to assess relevant epitopes. Most approaches used to reduce I-R damage are inflammatory. Therefore, many pharmacological effects of the peroxidative type have not been considered, thereby establishing the grouping of the said markers and targets of oxidative damage. After elucidating the molecular targets implicated in their physiopathology, a realistic approach in all pathology therapies is a pragmatic approach in all pathology therapies. The direct modulation of calcium overload in the cytosol can be a common strategy and, undoubtedly, a key element in stabilizing cellular pH. By contrast, the uptake of ROS mainly via scavenger molecules that may reduce damage can also be an exciting alternative.

11. Conclusion

I-R injury caused by the interruption of oxygen flow and the consecutive restoration of oxygen concentration, which is known as reperfusion, is still poorly understood [279]. These therapeutic targets can be a great alternative for modulating I-R injury. Reducing oxidative damage in cells could address several pathologies associated with I-R. Redox signaling is one of the processes with several therapeutic targets. However, most interventions present extremely ambiguous pharmacodynamics [280]. Thus, it is essential to elucidate the specific molecular mechanisms by which pharmacological interventions work. The mitochondria play a key role in the development of these cellular signaling [281]. Therefore, researchers are interested in approaches with correlated epitopes. Although the preclinical outcomes of targeted therapies are favorable, they have not yet been applied in clinical practice, and their adverse effects were not evaluated. Hence, pharmacological repositioning is a good alternative at present [282]. However, it is necessary to develop new active principles with a specific activity to resolve this pathology or to consider therapeutic combinations via in vitro and even in vivo tests [283]. Nevertheless, new active compounds with specific activity must dress to resolve pathologies or take advantage of the drug's antioxidant properties that can be considered adjuvant therapies in clinical settings. Proposing new therapy is necessary. This phenomenon is related to multiple pathologies and surgical procedures in oxidative stress and alterative to modulate ischemia-reperfusion.

Data Availability

We provide editable versions of our figures and editing platform publishing license.

Conflicts of Interest

The authors declare that they have no conflict of interest during the performance of this review, and it was conducted for academic purposes.

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Research Article

Nrf2 Deficiency Attenuates Testosterone Efficiency in Ameliorating Mitochondrial Function of the Substantia Nigra in Aged Male Mice

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Reduced testosterone level is a common feature of aging in men. Aging, as a risk factor for several neurodegenerative disorders, shows declined mitochondrial function and downregulated mitochondrial biogenesis and mitochondrial dynamics. Mitochondrial biogenesis and mitochondrial dynamics are crucial in maintaining proper mitochondrial function. Supplementation with testosterone is conducive to improving mitochondrial function of males during aging. Nuclear factor erythroid 2-related factor 2 (Nrf2), a regulator of redox homeostasis, is involved in the ameliorative effects of testosterone supplementation upon aging. To explore Nrf2 role in the effects of testosterone supplementation on mitochondrial function during aging, we studied the efficiency of testosterone supplementation in improving mitochondrial function of Nrf2 knockout- (KO-) aged male mice by analyzing the changes of mitochondrial biogenesis and mitochondrial dynamics. It was found that wild-type- (WT-) aged male mice showed low mitochondrial function and expression levels of PGC-1α, NRF-1\NRF-2, and TFAM regulating mitochondrial biogenesis, as well as Drp1, Mfn1, and OPA1 controlling mitochondrial dynamics in the substantia nigra (SN). Nrf2 KO aggravated the defects above in SN of aged male mice. Testosterone supplementation to WT-aged male mice significantly ameliorated mitochondrial function and upregulated mitochondrial biogenesis and mitochondrial dynamics, which were not shown in Nrf2 KO-aged male mice due to Nrf2 deficiency. Testosterone deficiency by gonadectomy (GDX) decreased mitochondrial function, downregulated mitochondrial biogenesis, and altered mitochondrial dynamics balance in young male mice. Supplementation with testosterone to Nrf2 KO-GDX mice only ameliorated the alterations above but did not reverse them to sham level. Nrf2 deficiency attenuated testosterone efficiency in ameliorating mitochondrial function in the SN of aged male mice through mitochondrial biogenesis and mitochondrial dynamics to some extent. Activation of Nrf2 might contribute to testosterone-upregulating mitochondrial biogenesis and mitochondrial dynamics in the SN during aging to produce efficient mitochondria for ATP production.

1. Introduction

Aging, as a risk factor for several neurodegenerative disorders including Parkinson's disease (PD) and Alzheimer's disease, shows mild to severe mitochondrial dysfunction. Mitochondrial dysfunction, such as the decreased oxidative phosphorylation, the increased reactive oxygen species (ROS), and oxidative damage, is found in aged subjects and subjects with aging-related neurodegeneration [1]. As an energy powerhouse, mitochondria are the main producer of ROS in the cells [2, 3]. While ROS are involved in normal cellular function, the overproduction of ROS disturbs redox homeostasis and contributes to the brain aging and aging-related neurodegeneration by oxidating the biomolecules

of neurons [2]. Due to obvious involvement in aging and aging-related neurodegeneration, the mitochondrion is identified as a major target for neuroprotection [4]. The reduction of oxidative damage to mitochondria by enhancing antioxidative capability and the preservation of the normal mitochondrial function via regulating mitochondrial biogenesis or mitochondrial dynamics have been proposed as strategies to mitigate aging and aging-related neurodegenerative disease [3, 5].

Mitochondrial biogenesis is a complex process, during which new mitochondria are formed from preexisting mitochondria through mechanisms involving interaction between the genetic systems of the nucleus and the mitochondria in the cells [4, 6]. Mitochondrial dynamics is another modification process of mitochondrial morphological state through alterations in mitochondrial fission and fusion activities [7]. Both of them are crucial in the maintenance of mitochondrial function to adapt to energy demands and in the regulation of cell metabolism and antioxidant defense [5, 8]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor controlling the levels of oxygen free radicals. Activation of the Nrf2-antioxidant response elements (ARE) can alleviate pathophysiological processes of neurodegeneration by reducing oxidative stress. Disruption of the Nrf2-ARE pathway results in an increased vulnerability to oxidant neurotoxin [9]. In addition to enhancing redox defense, the Nrf2-ARE pathway facilitates mitochondrial homeostasis and bioenergetics by regulating mitochondrial biogenesis [10] and maintaining balance of mitochondrial dynamics [11–13]. Thus, Nrf2 might be crucial target that affects the efficacy of the intervention strategy imposed on aging and age-related neurodegeneration through manipulating mitochondria.

Previous studies found that testosterone supplementation significantly ameliorates motor behavioral decline, enhances mesodopaminergic activity, and alleviates oxidative damage to the substantia nigra (SN) in aged male rats [14, 15]. Clinical data also showed that the supplementation with testosterone improves both motor symptom and nonmotor symptom of PD men to some extent [16]. The ameliorative effects of testosterone supplementation above might be related to the alteration of mitochondrial biogenesis and mitochondrial dynamics in the process of testosterone treatment [17, 18]. Gonadectomy to adult male rats decreases the gene expression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), which is a master regulator of mitochondrial biogenesis, in the hippocampus, and induces a significant reduction of mitochondrial DNA-encoded subunits and a marked elevation of oxidative damage, in the hippocampus and the SN [19, 20]. Orchiectomy upregulates mitochondrial fission protein and downregulates mitochondrial fusion protein in the rat myocardial infarction model, while testosterone replacement reverses these effects of orchiectomy on mitochondrial dynamics in cardiac muscles [17]. Thus, testosterone deficiency is an important factor inducing mitochondrial deficits and brain mitochondrial dysfunction in males [19, 20], the common early events of aging-related neurodegeneration [21]. Supplementation with testosterone in the aging process ameliorates aged-related brain mitochondrial dysfunction [18].

In vitro studies revealed that the oxidative stress status seems to play a crucial role in determining a neuroprotective or neurotoxic role of testosterone supplementation [22]. Nrf2, as a regulator of redox homeostasis and mitochondrial homeostasis, might determine the efficacy in the ameliorative effects of testosterone supplementation on aged-related brain mitochondrial dysfunction in males. Thus, to explore roles of Nrf2 in the effects of testosterone supplementation on the mitochondrial dysfunction in the aging process, the present study analyzed the alterations of mitochondrial function, mitochondrial biogenesis, and mitochondrial dynamics in the SN, as well as behavioral and dopaminergic parameters related to the SN, in testosterone-supplemented Nrf2 knockout-aged male mice. Furthermore, for comparison, young male mice were also included in the study to determine the effects of testosterone deficiency on the SN under the condition of Nrf2 knockout without aging factor interference.

2. Materials and Methods

2.1. Animals and Housing. Male Nrf2 wild-type (WT) ICR mice and Nrf2 knockout (KO) mice were kindly provided by academician Chunyan Li (Neurology Department, Second Hospital of Hebei Medical University). They were genotyped by PCR analysis of tail DNA and housed (3–4 per cage) in an air-conditioned room ($22 \pm 2^{\circ}$C) on a 12 h light/dark cycle with standard chow and water available ad libitum. All the experimental procedures followed the rules in the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" and were approved by the Committee of Institutional Animal Care and Use of Hebei Medical University.

2.2. Experiment 1. The aim of Experiment 1 is to investigate whether Nrf2 deficiency affected testosterone efficiency in ameliorating age-related changes related to the SN of male mice. Young KO (KO-young), aged KO (KO-aged), and testosterone propionate- (TP-) supplemented-aged KO (KO-aged-TP) mice were used. For KO-aged-TP mice, the mice received subcutaneous TP injection (1 mg/kg per day) at the age of 21 months and were sacrificed at the age of 23 months. KO-aged mice were subjected to the same treatment using sesame oil. WT mice were treated the same as KO mice and labeled as WT-young, WT-aged, and WT-aged-TP groups, respectively.

2.3. Experiment 2. To eliminate aging factors, young male mice were used to explore the effects of testosterone deficiency and testosterone replacement on the parameters analyzed above in Experiment 1. In Experiment 2, KO mice were divided into the sham-operated (KO-sham), the gonadectomized (KO-GDX), and the GDX with TP treatment (KO-GDX-TP) mice. For the GDX mice, anesthetized mice were castrated by the removal of the testes, epididymis, and epididymal fat under aseptic conditions. The sham-operated mice experienced the same surgical treatment

except for the bilateral orchiectomies [23]. For KO-GDX-TP mice, the castrated KO mice were subcutaneously injected with TP (1 mg/kg per day) at the age of 3 months and were sacrificed at the age of 5 months. KO-GDX mice experienced the same treatment as KO-GDX-TP mice with sesame oil instead of TP. Young WT mice were processed the same as KO mice and grouped into WT-sham, WT-GDX, and WT-GDX-TP groups.

2.4. Open-Field Test. The mice were back or tail-marked and handled for 5 days before the behavioral test. Open-field apparatus (75 × 75 × 30 cm) was placed in a quiet room with illumination of 20 lux. A digital video camera was set up above the arena to record mouse open-field activity. Each mouse was individually placed in the center of the open-field apparatus, and its five-minute open-field activity was recorded for further analysis. Based on a previous study [23], open-field activity related to total path length, exploratory behavior (walking, climbing, rearing, and sniffing), and grooming behavior (latency of grooming, number of grooming, and duration of grooming) was noted and scored in shorthand from the recorded videos by three independent observers (RC, XJ, and GZ) who were blind to the experimental plan. The behavioral data documented by them did not show any interobserver differences (ANOVA, NS).

2.5. Footprint Test. Walking gait of mouse was detected via footprint test. The apparatus for footprint test was a tunnel with 10 cm wide × 50 cm long × 10 cm high. The bottom of the tunnel was covered with white paper. During the experiment, each mouse with forepaws and hindpaws dipped in red and black ink, respectively, was placed at the brightly lit end of a tunnel, which was dark at its other end. The mouse walked down the tunnel, having a set of colored footprints on the white paper. After that, the paper with footprints was then removed for later analysis of walking gait defects. Three independent observers (RC, XJ, and GZ) blind to the experimental purpose measured the stride length of forelimb as well as hindlimb, and the overlap of footprints between forelimb and hindlimb at the right or left side. Stride length is the mean of the forelimb or hindlimb strides, and overlap is the mean of the distance between forelimb and hindlimb at the right or left side.

2.6. Sample Preparation. For LC-MS/MS, biochemical assay, mitochondrial membrane potential (MMP) detection, quantitative real-time PCR (qPCR), and western blot analysis, mice were sacrificed by decapitation. Their brains were removed quickly, and tissue blocks containing the SN or caudate putamen (CPu) were dissected on an ice-cold plate under stereomicroscopic observation. And then, they were immediately processed for reduced glutathione/oxidized glutathione (GSH/GSSG), malondialdehyde (MDA), H_2O_2, ATP, mitochondrial complexes, citrate synthase (CS), and MMP assays or stored at -80°C after freezing in liquid nitrogen for LC-MS/MS, qPCR, or western blotting on the experimental purposes. For immunohistochemistry (IH), mice were anesthetized and perfused transcardially by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The tissue blocks containing the SN were postfixed in the same fixative for 4 h at 4°C, dehydrated in graded ethanol, cleared in xylene, and then embedded in paraffin wax. For mitochondrial ultrastructure analysis, mice were anesthetized and perfused transcardially by a fixative containing 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M PB. The mouse brain was removed from the cranial cavity and further dissected under a stereomicroscope to collect the SN tissue block. After three washes in PB, the SN blocks were postfixed with 1% osmium tetroxide for 2 h, dehydrated in acetone, and then embedded in Araldite.

2.7. LC-MS/MS Assay. Tissue block containing CPu was weighed and homogenized in 80% acetonitrile containing 0.1% formic acid (5 μL) and then processed following a previous study [24]. The homogenates were centrifuged at 14,000g for 10 min at 4°C. The supernatants were collected and used to determine dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels as previously described [24]. LC separation was performed on an Agilent 1200 LC system (Agilent, Santa Clara, USA) using a Synergi Fusion-RP C18 column (50 mm × 3.0 mm, 4 μm) provided by Phenomenex. MS/MS detection was carried out using a 3200 QTRAP™ LC-MS/MS System (Applied Biosystems, Foster City, CA, USA). The multiple-reaction monitoring mode was used for quantification. The principal validation parameters of the LC-MS/MS are described in a previous study [24].

2.8. Biochemical Analysis

2.8.1. GSH/GSSG. The SN tissue blocks were homogenized in a solution provided by the GSH/GSSG kit (Code No. A061-1, Nanjing Jiancheng Bioengineering Institute, China) and then centrifuged at 14,000 × g for 15 min at 4°C. The supernatants from centrifuged homogenates were used to assess the GSH/GSSG ratio following the instructions of the GSH/GSSG kit. GSH and GSSG in the samples were first made to react with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) to produce a colored reagent. Then, the 2-vinylpyridine reagent was added to the sample supernatant to ensure that GSSG is the only form of glutathione that reacts with the DTNB reagent. Lastly, the GSSG level was subtracted from the total GSH levels to yield the concentration of reduced GSH. GSH and GSSG levels in the SN were assessed at 412 nm by a spectrophotometer.

2.8.2. MDA Levels. MDA was detected based on its reaction with thiobarbituric acid (TBA). For the measurement of MDA levels in the SN, tissue blocks were weighed and homogenized in 10 times (w/v) ice-cold 0.1 M PB at pH 7.4. The homogenates were centrifuged at 3000 rpm for 10 min, and the supernatant was processed according to the instructions of an MDA kit (Code No. A003-1, Nanjing Jiancheng Bioengineering Institute, China). The absorbance was read at 532 nm by a spectrophotometer.

2.8.3. H_2O_2 Levels. For the detection of H_2O_2 in the mitochondria of the SN, the mitochondria were isolated using the tissue mitochondria isolation kit (Code C3606, Beyotime

Institute of Biotechnology, China). In brief, SN tissue was homogenized in ice-cold buffer (10 mM HEPES, pH 7.5, including 200 mM mannitol, 70 mM sucrose, 1.0 mM EGTA, and 2.0 mg/mL serum albumin) and centrifuged at 1000g at 4°C for 10 min. The supernatant was centrifuged again at 3500g at 4°C for 10 min to collect a mitochondrial pellet. The levels of H_2O_2 in the mitochondria were measured spectrophotometrically at 415 nm according to the protocol of the detection kit (Cat. No. AKAO009M, Beijing Boxbio Science & Technology, China).

2.8.4. ATP Levels. For detection of ATP levels, the mitochondria were isolated using the Tissue Mitochondria Isolation Kit (Code C3606, Beyotime Institute of Biotechnology, China). ATP levels were measured in isolated mitochondria using an ATP colorimetric assay kit following the manufacturer's instructions (A095-1-1, Nanjing Jiancheng Biotechnology Institute, China). Total mitochondrial protein samples were incubated with the ATP reaction mixture at 37°C for 30 min and detected at 636 nm using a microplate reader (BioTek Instruments Inc., Highland Park, USA) [25].

2.8.5. Mitochondrial Complex Activities. Mitochondria from the SN tissue block were isolated according to the protocol of the detection kits. The activities of mitochondrial complexes I, II, III, IV, and V were measured spectrophotometrically using detection kits for complex I (Cat. No. AKOP005M) at 340 nm, complex II (Cat. No. AKOP006M) at 605 nm, complex III (Cat. No. AKOP007M) at 550 nm, complex IV at 550 nm (Cat. No. AKOP008M, Beijing Boxbio Science & Technology), or complex V at 340 nm (A089-5-1, Nanjing Jiancheng Institute of Biotechnology, China) according to manufacturer's specifications.

2.8.6. Citrate Synthase Assay. CS activity in the SN was estimated based on the reduction of 5,5′-dithio-bis-(2-nitrobenzoic acid) following the specifications of a citrate synthase kit (Code No. A108, Nanjing Jiancheng Bioengineering Institute, China). The SN tissue blocks were firstly homogenized in 0.01 M ice-cold phosphate buffer saline (PBS, pH 7.4) and centrifuged at 14,000 × g for 15 min at 4°C. And then, the CS activities in the supernatant were measured spectrophotometrically based on the instructions of the citrate synthase kit. Absorption values were obtained spectrophotometrically at 412 nm.

2.9. Mitochondrial Membrane Potential Detection. MMP in the SN was detected by the Rhodamine 123 (Rh123) fluorescence method as described previously [26]. The SN tissue block was homogenized in a balanced salt solution and filtered through a nylon mesh screen. The cells were harvested routinely. Following two washes with ice-cold PBS, the cells were incubated in Rh123 solution (10 μg/mL) at 37°C for 30 min. After being washed and resuspended in 1 mL PBS, the cells were immediately analyzed by flow cytometry (excitation/emission wavelengths, 488/534 nm). MMP was determined by analyzing the changes in Rh123 fluorescence intensity.

2.10. Quantitative Real-Time PCR Analysis. Total RNA from SN tissue block was isolated according to the manufacturer's protocol using TRizol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA was reverse-transcribed using random primers to obtain the first-strand cDNA template. Then, qPCR was performed with 20 μL reaction solution containing 0.8 μL cDNA (diluted 1 : 10), 2 μL-specific primers, and 2x GoTaq® Green Master Mix (Promega, USA). PCR was performed as follows: an initial cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s. The melting curves of the PCR products were analyzed to confirm the specificity of amplification. Gene expression of *PGC-1α*, nuclear respiratory factor 1 (*NRF-1*), *NRF-2*, mitochondrial transcription factor (*TFAM*), mitochondrial fission protein dynamin-related protein (*Drp1*), mitochondrial fusion protein mitofusin 1 (*Mfn1*), or optic atrophy protein 1 (*OPA1*) was analyzed using *GAPDH* as the internal control. For all samples, qPCR was performed in triplicate. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method. The sets of primers were as follows: *PGC-1α* (5′-GAAAGGGCC AAACAGAGAGA-3′ and 5′-GTAAATCACACGGCGC TCTT-3′), *NRF-1* (5′-TGGAGTCCAAGATGCTAATG-3′ and 5′-AGAGCTCCATGCTACTGTTC-3′), *NRF-2* (5′-TCAGTGACTCGGAAATGGAG-3′ and 5′-TTCACGCAT AGGAGCACTGT-3′), *TFAM* (5′-CAGGAGGCAAAGGA TGATTC-3′ and 5′-CCAAGACTTCATTTCATTGTCG-3′), *Drp1* (5′-CAGGAATTGTTACGGTTCCCTAA-3′ and 5′-CCTGAATTAACTTGTCCCGTGA-3′), *Mfn1* (5′-AACT TGATCGAATAGCATCCGAG-3′ and 5′-GCATTGCAT TGATGACAGAGC-3′), *OPA1* (5′-GATGACACGCTCTC CAGTGA-3′ and 5′-TCGGGGCTAACAGTACAACC-3′), and *GAPDH* (5′-ACTCTTCCACCTTCGATGCC-3′ and 5′ -TCTTGCTCAGTGTCCTTGCT-3′). Accession numbers of the genes for primers are listed in Table S1.

2.11. Analysis of mtDNA Copy Number. Total DNA was extracted from the SN tissue blocks using an Animal Tissue Genomic DNA kit (ZP307-2, ZOMANBIO, China) according to the manufacturer's protocol. Mitochondrial DNA (mtDNA) copy number was determined by quantifying *16S rRNA* from mtDNA and nuclear-encoded hexokinase 2 (*HK2*) gene expression via qPCR. qPCR was carried out with 1 μL of sample DNA (diluted 1 : 10), 2 μL of each specific primer, and 2x All-in-OneTM qPCR Mix (GeneCopoeia Inc., USA) in a final volume of 10 μL. The primers for *16S rRNA* and *HK2* were used as follows: *16S rRNA* (5′-CCGC AAGGGAAAGATGAAAGAC-3′ and 5′-TCGTTTGGTTT CGGGGTTTC-3′) and *HK2* (5′-GCCAGCCTCTCCTGAT TTTAGTGT-3′ and 5′-GGGAACACAAAAGACCTCTTC TGG-3′). Accession numbers of the genes for primers are listed in Table S1. PCR was performed as follows: an initial cycle at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. The melting curves of the PCR products were analyzed to confirm the specificity of amplification. qPCR was performed in triplicate. Relative mtDNA copy number was calculated by the ratio between *16S rRNA* and *HK2* genes using the $2^{-\Delta\Delta Ct}$ method.

2.12. Western Blot Analysis. The SN or CPu tissue block was homogenized in radioimmunoprecipitation assay buffer containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors (phenylmethanesulfonyl fluoride 100 μg/mL, aprotinin 30 μg/mL, and sodium orthovanadate 1 mM) and sonicated for 4 × 10 s. After centrifugation at 12,000g for 20 min at 4°C, the supernatant was collected and stored at −80°C for detection of target protein based on the study needs. The pellets were homogenized in ice-cold lysis buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, and 0.1 mM EGTA) for 15 min. The homogenate was centrifuged at 12,000g for 10 min at 4°C, and the supernatant was collected for detection of nuclear Nrf2 protein. Samples from the supernatant were diluted in 5x sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS-PAGE on a 10% gel and transferred to a PVDF membrane (Millipore). The membrane was incubated for 2 h with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST). After being rinsed thrice with TBST, the membrane was incubated overnight with rabbit anti-tyrosine hydroxylase (TH) (1 : 10,000, Abcam), rabbit anti-dopamine transporter (DAT) (1 : 1000, Sigma), rabbit anti-PGC-1α (1 : 1000, Abcam), rabbit anti-NRF-1 (1 : 1000, ABclonal), rabbit anti-NRF-2 (1 : 1000, ABclonal), rabbit anti-TFAM (1 : 1000, GeneTex), rabbit anti-Drp1 (1 : 1000, Cell Signaling Technology), rabbit anti-pDrp1-S616 (1 : 1000, Affinity), rabbit anti-Mfn1 (1 : 1000, arigo), rabbit anti-OPA1 (1 : 1000, GeneTex), mouse anti-Nrf2 (1 : 500, Santa Cruz Biotechnology), rabbit anti-heme oxygenase 1 (HO-1, 1 : 300, Affinity), rabbit anti-β-actin (1 : 10,000, ABclonal), or rabbit anti-H3 (1 : 1000, Arigo) antibody at 4°C according to the study purposes. After three washes, the membrane was incubated for 1 h in IRDye® 800-conjugated goat anti-rabbit (1 : 10,000; Rockland) or anti-mouse (1 : 5,000; Rockland) second antibody. The bands were scanned by an Odyssey infrared scanner (LI-COR Biosciences). The densitometry values of the individual protein were normalized with respect to those of β-actin or H3, which was used as the endogenous control. For detecting the oxidation of mitochondrial proteins, mouse anti-3-nitrotyrosine (3-NT) antibody (1 : 1000, Santa Cruz Biotechnology) or rabbit anti-VDAC (1 : 1000, ABclonal) was used to incubate the PVDF containing the electroblotted proteins from isolated mitochondrial fractions. After performing the same western blot procedures as described before, 3-NT densitometry values were normalized with those of VDAC that was used as mitochondrial endogenous control.

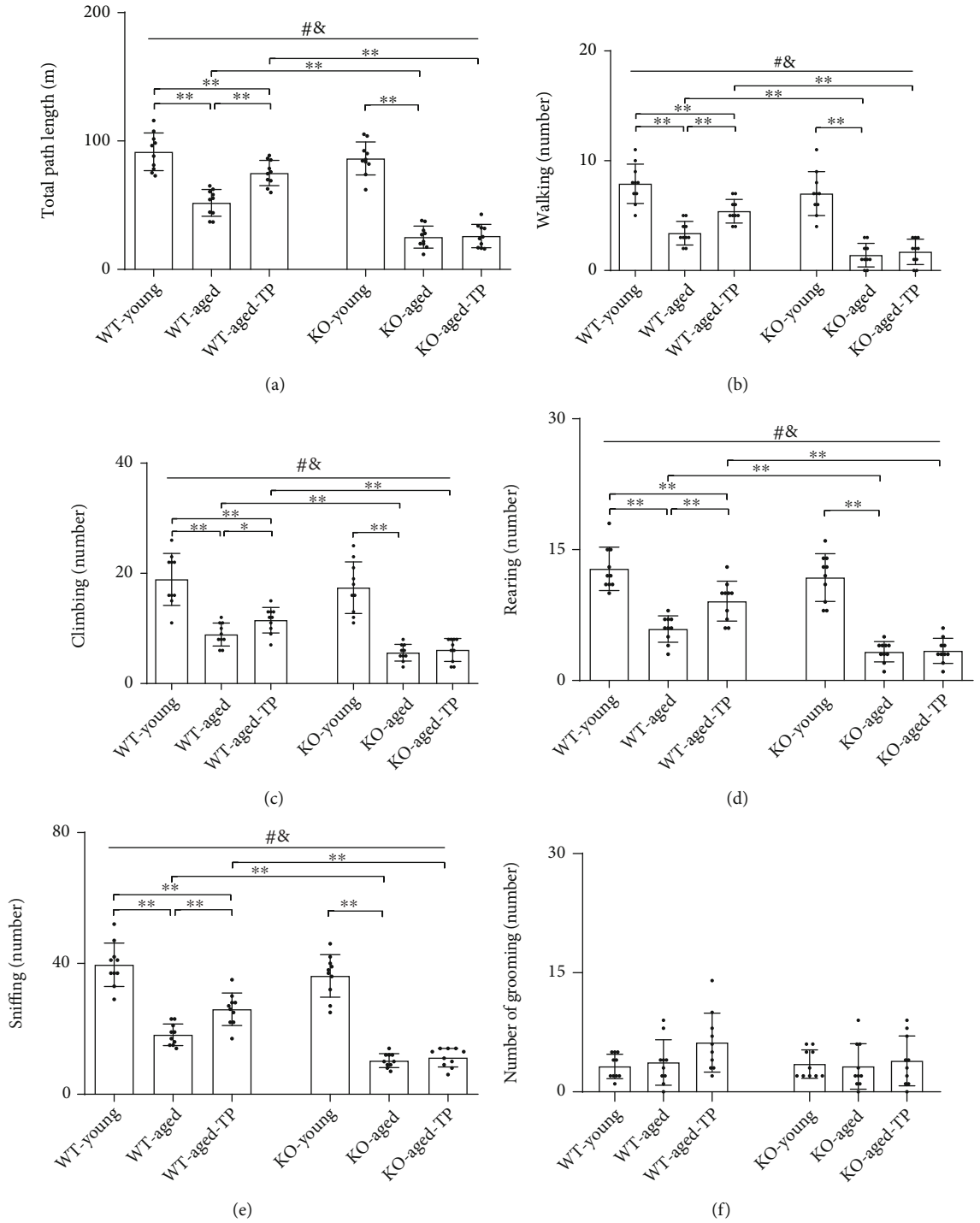
2.13. Immunohistochemistry. 5 μm coronal sections sliced from paraffin-embedded SN tissue blocks were mounted on the slides. After deparaffinization and hydration, the sections were processed for antigen retrieval, inactivation of endogenous peroxidase activity, and incubation in normal serum. Subsequently, the sections were incubated with mouse anti-3-nitrotyrosine (3-NT) antibody (1 : 100, Santa Cruz Biotechnology) overnight at 4°C. After washing, the sections were incubated with biotinylated goat anti-mouse IgG (1 : 500) for 2 h at room temperature. Following incubation at room temperature in horseradish peroxidase-conjugated streptavidin (1 : 500) for 1 h, the sections were stained for 5 min in a solution containing 0.05% diaminobenzidine and 0.03% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). A computer-assisted image analysis system (Image-Pro Plus 6.0) was used to measure the average optical density (AOD) and the number of 3-NT immunoreactive (3-NT-ir) positive cells in the SN.

2.14. Mitochondrial Ultrastructure Analysis. Ultrathin sections (70 nm) were obtained with a microtome (UC-7, Leica, Austria). After staining with uranyl acetate (10 min) and lead citrate (5 min), the sections were examined under a transmission electron microscope (Hitachi HT7800, Japan) operated at 80 kV. For the electron microscopy (EM) image analyses, the mitochondrial number was counted using Image-Pro Plus 6.0 (Media Cybernetics, USA) at ×3000 magnification, and the mitochondrial ultrastructure was analyzed at ×25,000 magnification.

2.15. Statistics. The data are presented as the mean ± SD. All the data were analyzed by a two-way ANOVA. If the two-way ANOVA was significant, we performed planned comparisons using one-way analysis of variance (one-way ANOVA) for the comparison of treatment effect among same genotype or using Student's t-test for the comparison of genotype effect among same treatment. For one-way ANOVA, Levene's test was applied to test of homogeneity of variance. If homogeneity of variance is equal (the significance of Levene's test is greater than 0.05), go to the homogeneity of variance tests (F-statistic), where $P < 0.05$, followed by Tukey's honestly significant difference (Tukey's HSD) post hoc test for multiple comparisons. If homogeneity of variance is unequal (the significance of Levene's test is less than 0.05), go to the test of Welch's F test (F'-statistic), where $P < 0.05$; the post hoc test between groups were done using the Games-Howell procedure. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Nrf2 Deficiency Attenuated Testosterone Efficiency in Improving Open-Field Activity and Walking Gait of Aged Male Mice. We first performed open-field test and footprint test to observe the behavioral changes of experimental mice among KO-young, KO-aged, and KO-aged-TP, as well as WT-young, WT-aged, and WT-aged-TP groups. Analysis to them revealed declined total path length, walking, climbing, rearing, and sniffing, as well as decreased stride length and increased overlap of footprints in WT-aged mice and KO-aged mice compared with corresponding control of their own ($P < 0.01$, Figures 1(a)–1(e) and 1(i)–1(l)). Further reduction in total path length, walking, climbing, rearing, sniffing, and stride length, as well as further increment in overlap of footprints, was found in KO-aged mice compared with WT-aged mice ($P < 0.01$). The male mice between WT-young and KO-young groups did not show significant difference in above behavioral



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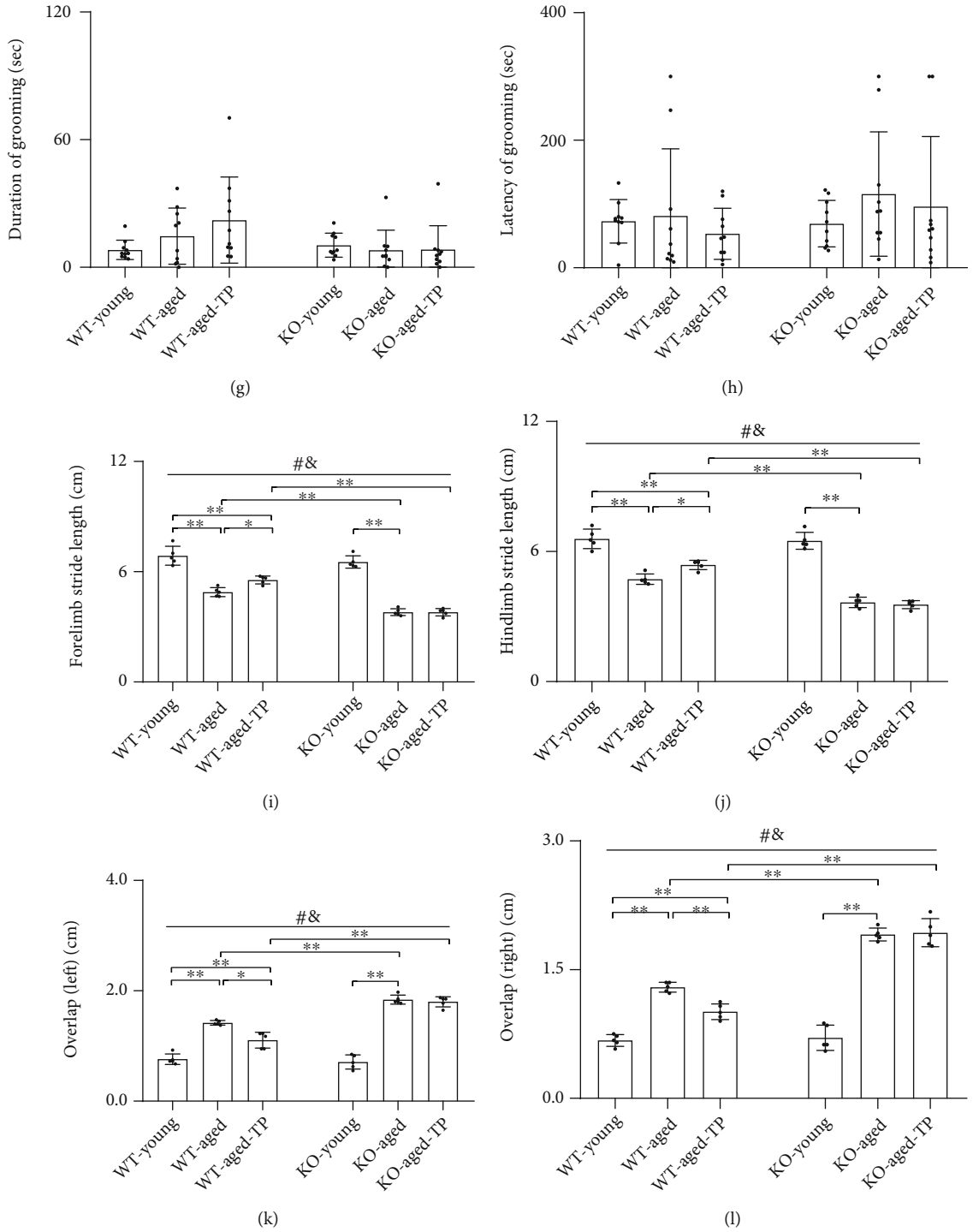


FIGURE 1: Effects of TP supplementation on open-field activity and walking gait of Nrf2 KO-aged male mice: (a) total path length, (b) walking, (c) climbing, (d) rearing, (e) sniffing, (f) number of grooming, (g) duration of grooming, (h) latency of grooming, (i) forelimb stride length, (j) hindlimb stride length, (k) overlap of left footprints, and (l) overlap of right footprints. Data were presented as mean ± SD; $n = 10$ for open-field test; $n = 5$ for footprint test. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

parameters. TP supplementation to WT-aged mice significantly increased their total path length, walking, climbing, rearing, sniffing, and stride length and decreased overlap of footprints. These effects by TP were not shown in KO-aged-TP mice. There was no significant intergroup difference among KO-young, KO-aged, KO-aged-TP, WT-young, WT-aged, and WT-aged-TP mice in grooming behavior (Figures 1(f)–1(h)). TP supplementation ameliorated open-field activity and walking gait in WT-aged male mice, but not in KO-aged male mice.

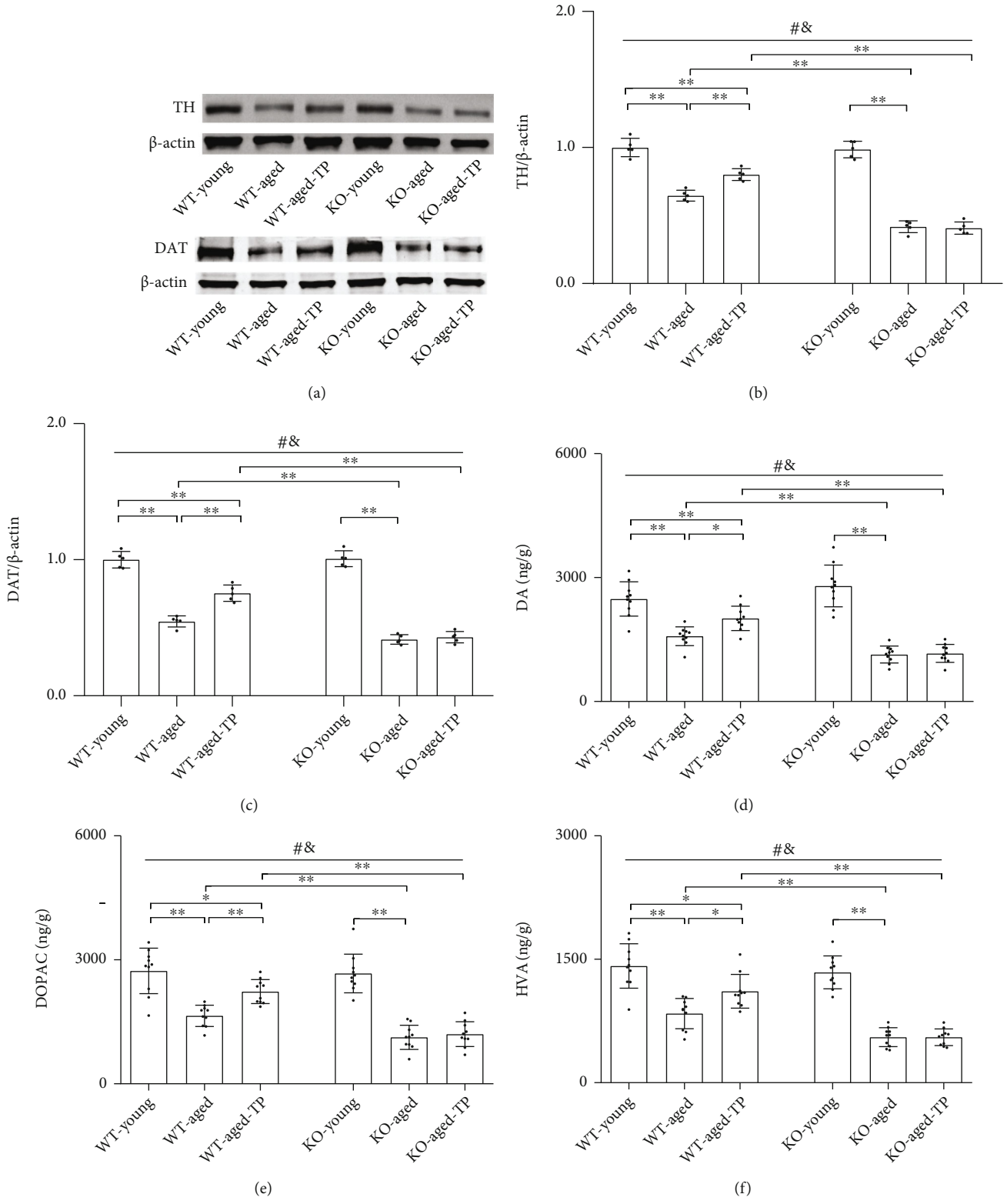
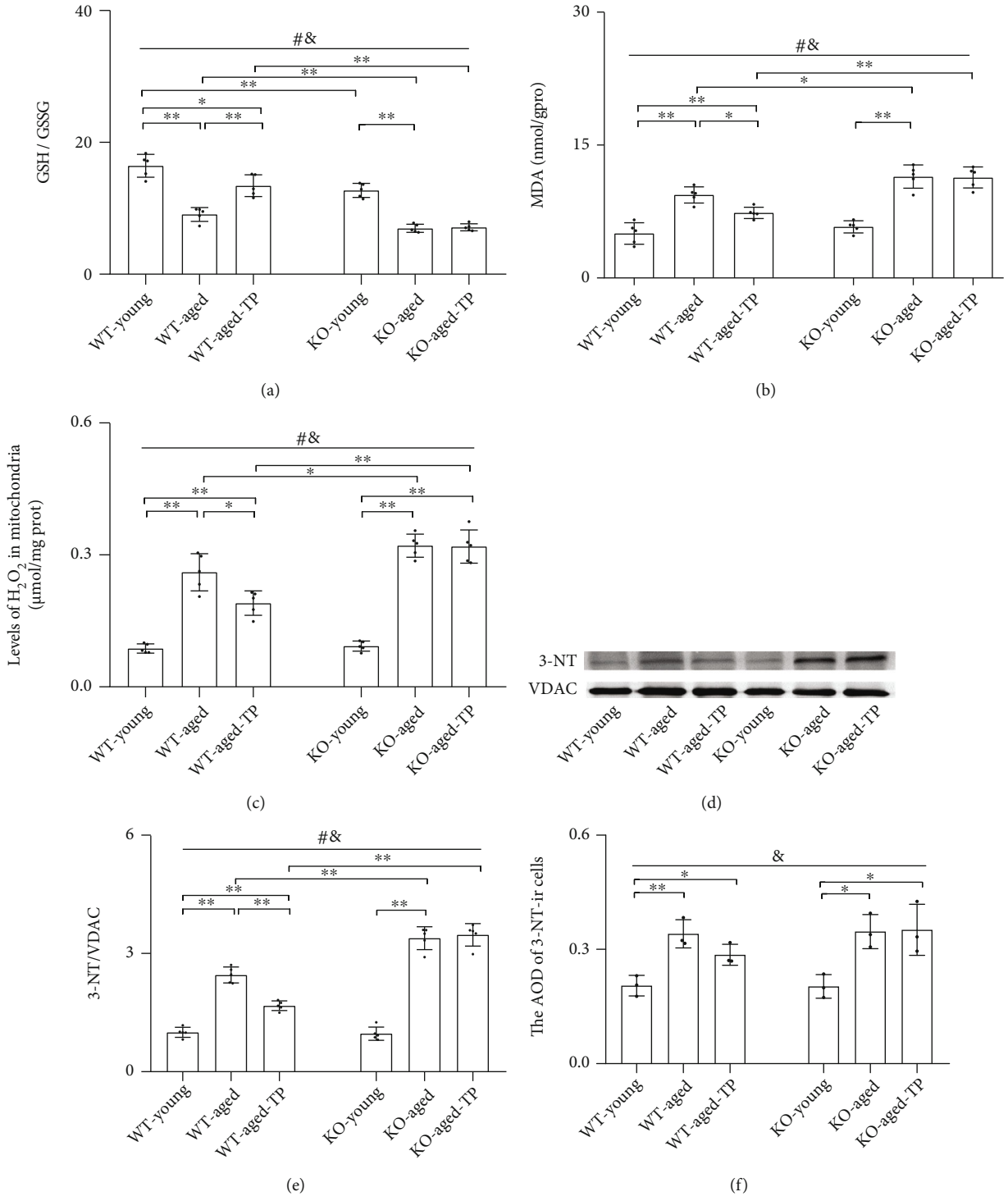


FIGURE 2: Effects of TP supplementation on dopaminergic activity in the caudate putamen of Nrf2 KO-aged male mice. (a, b) TH and (a, c) DAT were detected by immunoblotting. (d) DA, (e) DOPAC, and (f) HVA were measured by LC-MS/MS assay. Data were presented as mean ± SD; $n = 5$ for TH and DAT; $n = 10$ for DA, DOPAC, and HVA. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.



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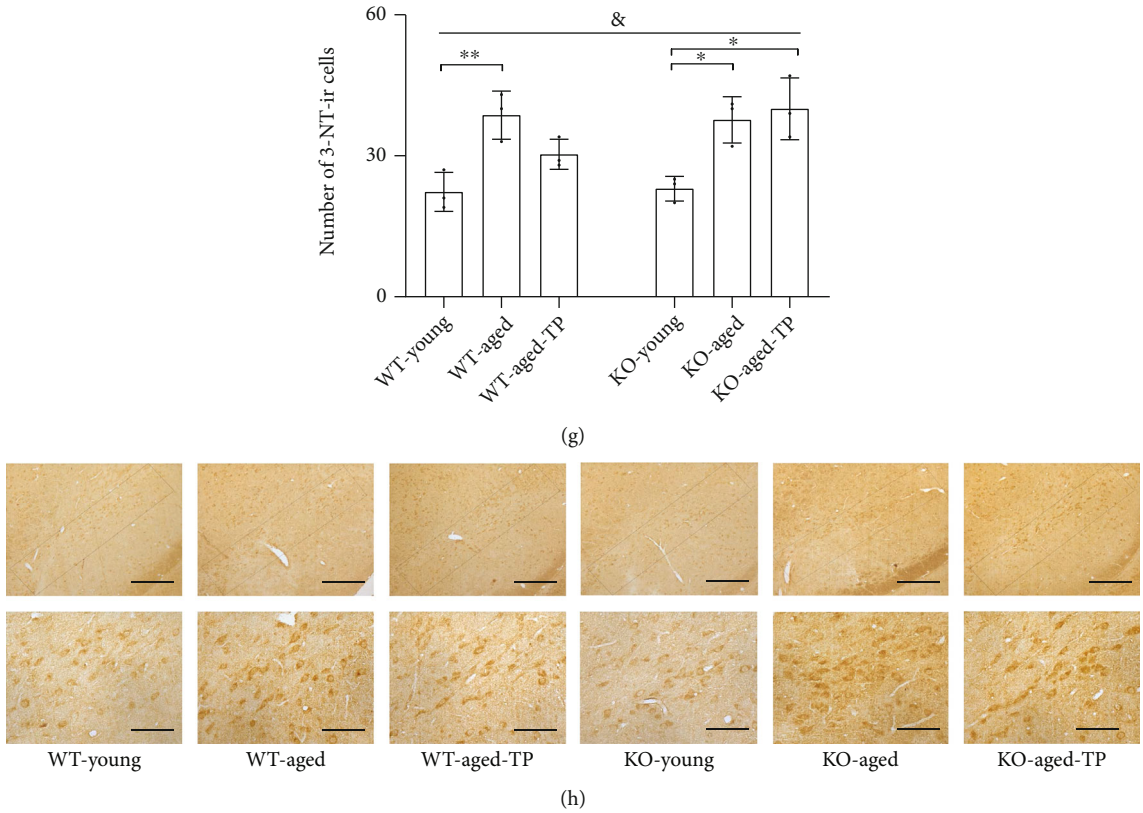
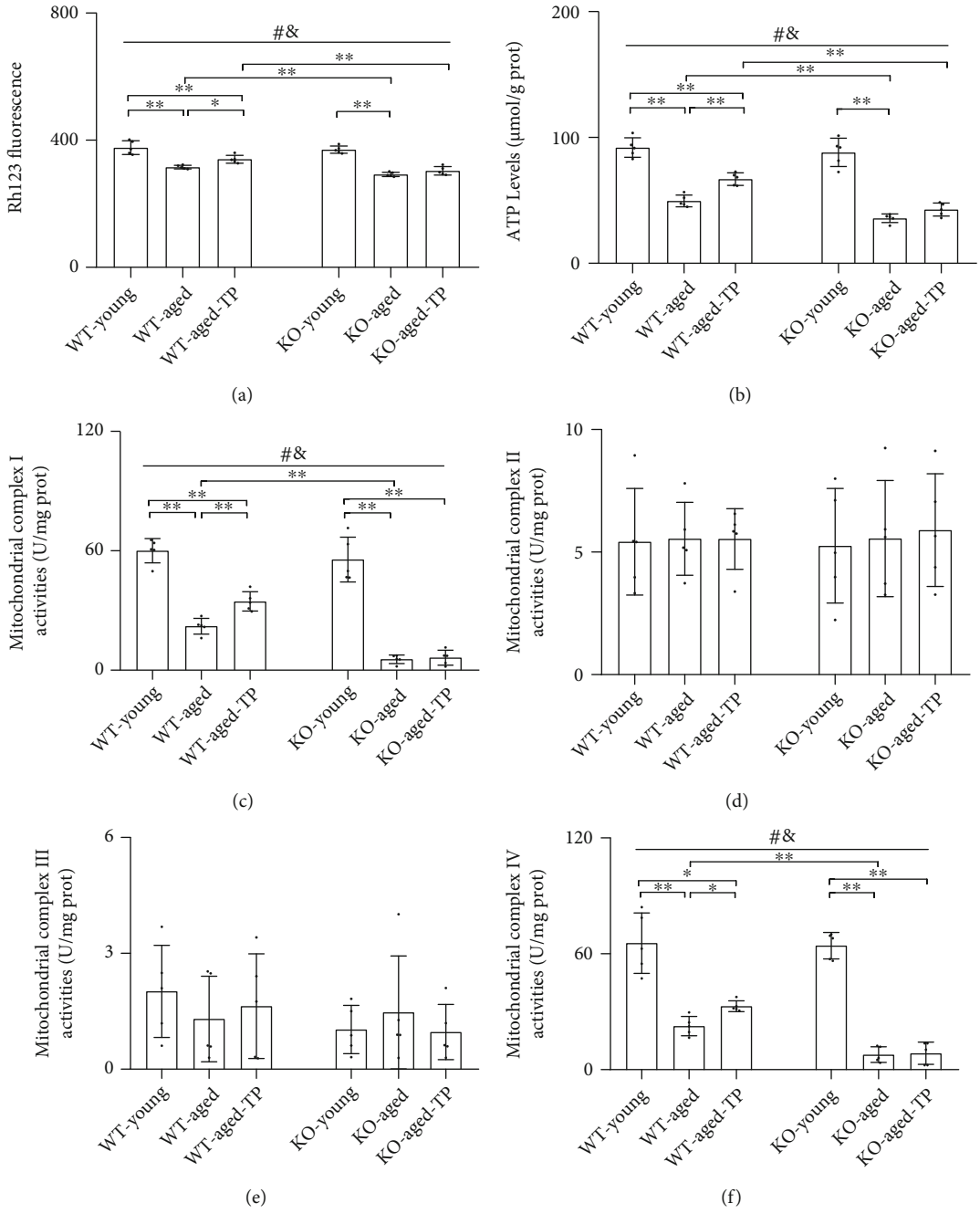


Figure 3: Effects of TP supplementation on oxidative balance in the substantia nigra of Nrf2 KO-aged male mice. (a) GSH/GSSG, (b) MDA, and (c) mitochondrial H_2O_2 were assessed by spectrophotometry. (d, e) Mitochondrial 3-NT was measured by immunoblotting; (f–h) 3-NT in the SN was detected by immunohistochemistry. Data were presented as mean ± SD; $n = 5$ for GSH/GSSG, MDA, mitochondrial H_2O_2, and mitochondrial 3-NT; $n = 3$ for 3-NT immunohistochemistry. Scale bars = 50 μm (lower panel); scale bars = 200 μm (upper panel). $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.2. Nrf2 Deficiency Attenuated Testosterone Efficiency in Enhancing Nigrostriatal Dopaminergic Activity of Aged Male Mice. The SN is a brain region rich in dopaminergic neurons. It controls motor behavior and exploratory behavior through a target region in the CPu [27–29]. Thus, we analyzed the altered status of nigrodopaminergic neurons of aged experimental mice under the Nrf2 deficiency by detecting TH and DAT expression, as well as dopaminergic neurochemical content in the CPu. The levels of TH and DAT, as well as DA, DOPAC, and HVA in the CPu, were lower in aged male mice of both genotypes than their corresponding young control mice ($P < 0.01$), and they were much lower in KO-aged mice than in WT-aged mice ($P < 0.01$, Figures 2(a)–2(f)). There was no difference between WT-young mice and KO-young male mice in the levels of TH and DAT, as well as DA, DOPAC, and HVA in the CPu. Administration of TP significantly increased the expression levels of TH and DAT, as well as DA, DOPAC, and HVA in the CPu of WT-aged mice. Increased parameters above by TP were not observed in the CPu of KO-aged-TP mice. TP supplementation increased TH and DAT expression, as well as dopaminergic neurochemical content in the CPu of WT-aged male mice, not in KO-aged male mice.

3.3. Nrf2 Deficiency Attenuated Testosterone Efficiency in Ameliorating Oxidative Balance in the SN of Aged Male Mice. Oxidative balance is critically involved in the aging process; therefore, in the SN, we next detected important parameters related to oxidative balance, i.e., GSH/GSSG ratio (a major biomarker of redox status in biological systems), MDA (a marker of ROS-mediated cell membrane damage), and 3-NT (an oxidative stress biomarker of protein nitration) in the tissue, as well as the levels of H_2O_2 and 3-NT in the mitochondria. Significantly low GSH/GSSG ratio and high MDA and mitochondrial H_2O_2 levels in the SN, as well as increased 3-NT levels of the cells and mitochondria in the SN, were found in aged male mice of two genotypes compared with corresponding young male mice (Figures 3(a)–3(h)). Nrf2 KO further lowered GSH/GSSG ratio and elevated MDA levels as well as mitochondrial H_2O_2 and 3-NT levels of aged male mice. Compared with WT-young mice, KO-young mice showed a reduced GSH/GSSG ratio in the SN ($P < 0.01$). Supplementation with TP significantly increased GSH/GSSG ratio and decreased MDA levels as well as mitochondrial H_2O_2 and 3-NT levels of WT-aged male mice. A slight, nonsignificant reduction of 3-NT levels of cells was detected in the SN of WT-aged-TP mice relative to WT-aged mice (Figures 3(f)–3(h)).



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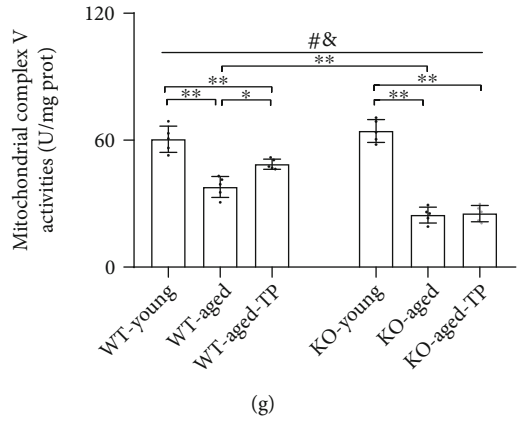


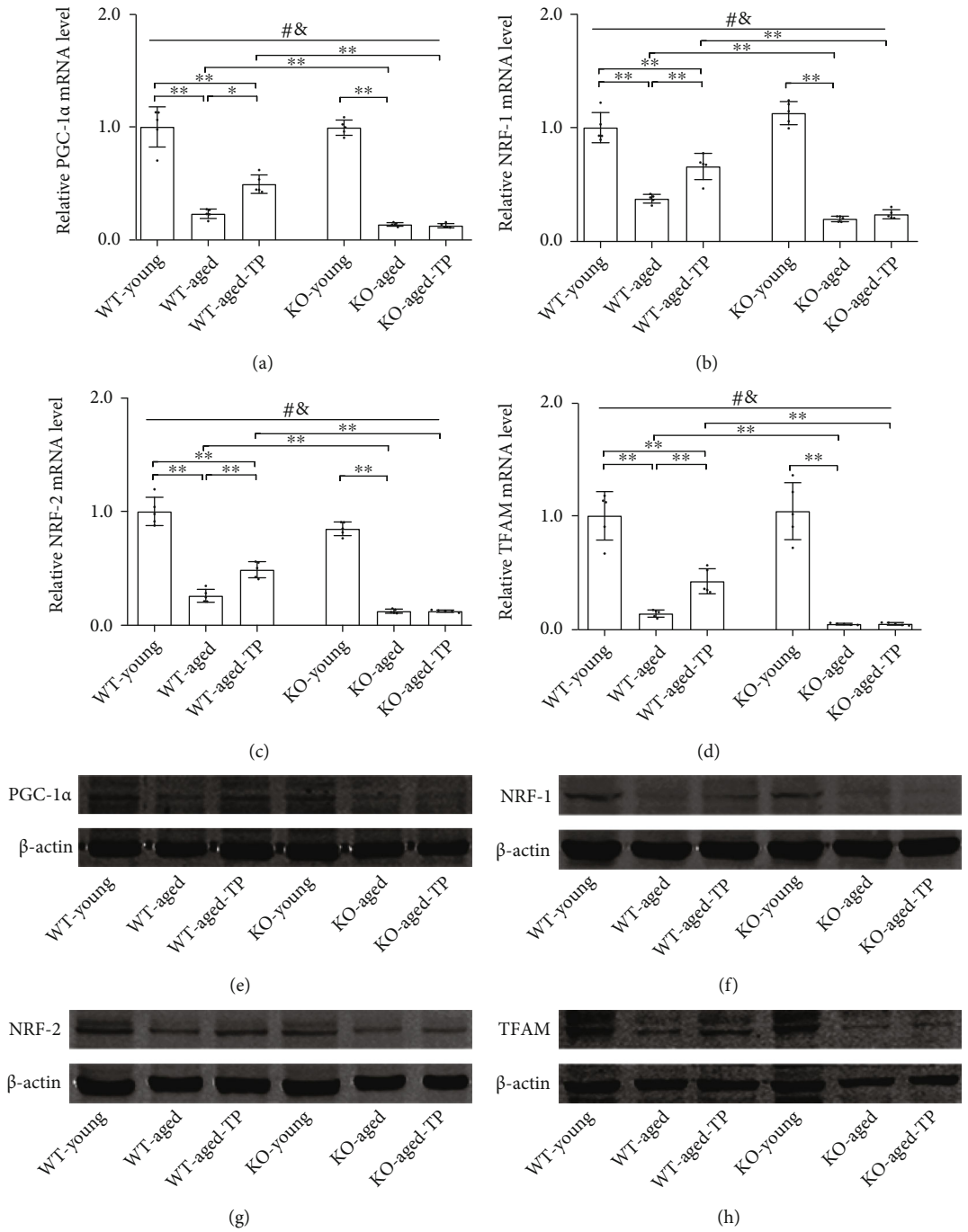
Figure 4: Effects of TP supplementation on mitochondrial function in the substantia nigra of Nrf2 KO-aged male mice. (a) Mitochondrial membrane potential was revealed using the Rh123 fluorescence method. (b) Mitochondrial ATP level, (c) mitochondrial complex I activity, (d) mitochondrial complex II activity, (e) mitochondrial complex III activity, (f) mitochondrial complex IV activity, and (g) mitochondrial complex V activity were revealed by spectrophotometry. Data were presented as mean ± SD; $n = 5$. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Increased GSH/GSSG ratio as well as decreased MDA, mitochondrial H_2O_2, and 3-NT by TP was not shown in KO-aged-TP mice. TP supplementation ameliorated oxidative balance in the SN of WT-aged male mice, not in KO-aged male mice.

3.4. Nrf2 Deficiency Attenuated Testosterone Efficiency in Ameliorating Mitochondrial Function in the SN of Aged Male Mice. As an early event in aging and age-related neurodegenerative diseases, mitochondrial dysfunction leads to insufficient energy and excessive ROS. Healthy neuronal status depends on proper energy supply and oxidative balance. So we further assessed the effects of Nrf2 deficiency on mitochondrial function in the SN of aged male mice during TP supplementation by measuring MMP, ATP levels, and mitochondrial complex activities. Decreased MMP and mitochondrial ATP levels, as well as the activities of mitochondrial complexes I, IV, and V, were detected in the SN of aged male mice of two genotypes compared with corresponding young male mice ($P < 0.01$, Figures 4(a)–4(c), 4(f), and 4(g)). Nrf2 KO further decreased them in the SN of aged male mice ($P < 0.01$). No significant difference in MMP and mitochondrial ATP levels, as well as activities of mitochondrial complexes I, IV, and V, was found between WT-young and KO-young mice. Supplementation with TP increased MMP and mitochondrial ATP levels, as well as the activities of mitochondrial complexes I, IV, and V in the SN of WT-aged male mice. Increased MMP and mitochondrial ATP levels, as well as the activities of mitochondrial complexes I, IV, and V, were not shown in the SN of KO-aged-TP mice. There was no significant intergroup difference among KO-young, KO-aged, KO-aged-TP, WT-young, WT-aged, and WT-aged-TP mice in the activities of mitochondrial complexes II and III (Figures 4(d) and 4(e)). TP supplementation ameliorated mitochondrial function in the SN of WT-aged male mice, but not in KO-aged male mice.

3.5. Nrf2 Deficiency Attenuated Testosterone Efficiency in Increasing PGC-1α and Its Downstream Target Expression in the SN of Aged Male Mice. Based on the decreased mitochondrial biogenesis signaling in aging process, the improved mitochondrial function in aging via the induction of mitochondrial biogenesis [30], and the above-found effects of Nrf2 deficiency on mitochondrial function of aged male mice during TP supplementation, we next examined the altered expression of key inducer and effectors of mitochondrial biogenesis, namely, PGC-1α, NRF-1, NRF-2, and TFAM in the SN of experimental mice. Aged male mice of both genotypes showed decreased mRNA levels of *PGC-1α*, *NRF-1*, *NRF-2*, and *TFAM* in the SN relative to respective young control ($P < 0.01$, Figures 5(a)–5(d)). Nrf2 KO further reduced their mRNA levels in the SN of aged male mice ($P < 0.01$). *PGC-1α*, *NRF-1*, *NRF-2*, and *TFAM* mRNA levels were not significantly different in the SN of KO-young mice relative to WT-young mice. Supplementation with TP significantly increased *PGC-1α*, *NRF-1*, *NRF-2*, and *TFAM* mRNA levels in the SN of WT-aged male mice. There was no difference between KO-aged mice and KO-aged-TP mice in the expression levels of *PGC-1α* and its downstream targets. Immunoblotting data from PGC-1α, NRF-1, NRF-2, and TFAM agreed with their mRNA changes (Figures 5(e)–5(l)). TP supplementation increased the expression levels of PGC-1α and its downstream targets in the SN of WT-aged male mice, but not in KO-aged male mice.

3.6. Nrf2 Deficiency Attenuated Testosterone Efficiency in Increasing Mitochondrial Content in the SN of Aged Male Mice. Since stimulation of mitochondrial biogenesis is accompanied by increased mitochondrial content, so following, we investigated the effects of Nrf2 deficiency on mitochondrial content in the SN of aged mice supplemented with TP through detecting CS activity (a mitochondrial matrix enzyme), mtDNA copy number, and mitochondrial number. CS activity, mtDNA copy number, and



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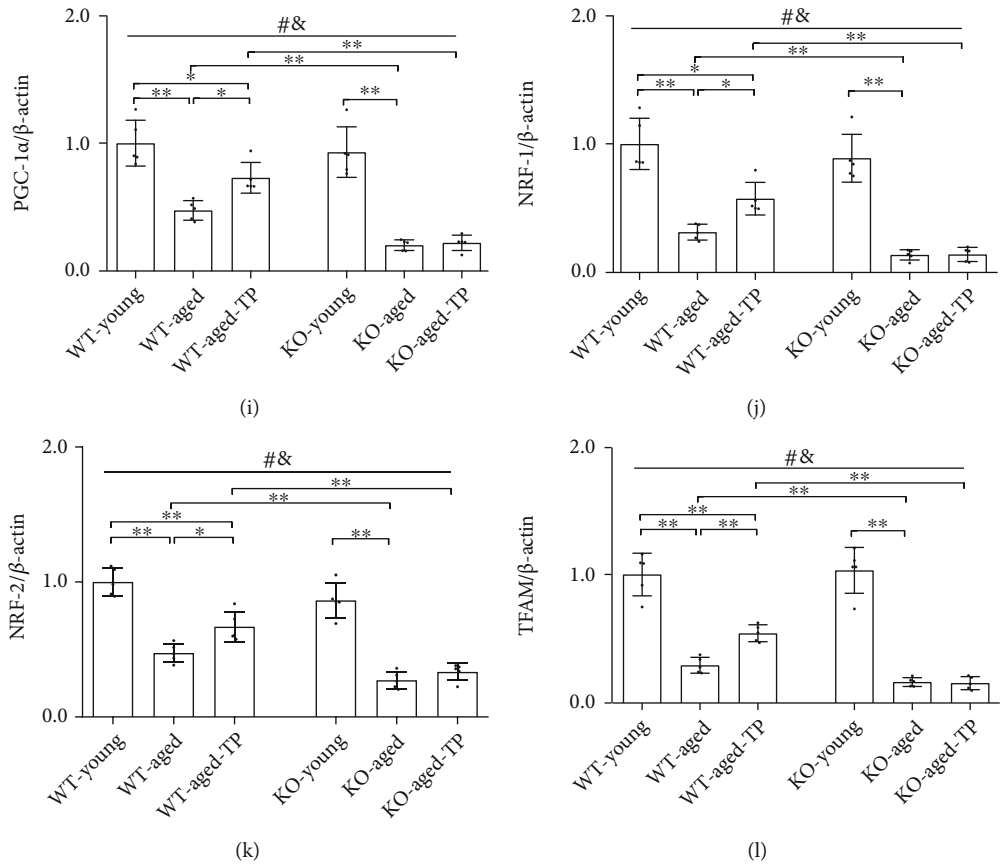


Figure 5: Effects of TP supplementation on mitochondrial biogenesis in the substantia nigra of Nrf2 KO-aged male mice. (a) *PGC-1α*, (b) *NRF-1*, (c) *NRF-2*, and (d) *TFAM* mRNAs were revealed by qPCR. (e, i) PGC-1α, (f, j) NRF-1, (g, k) NRF-2, and (h, l) TFAM proteins were detected by immunoblotting. Data were presented as mean ± SD; $n = 5$. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

mitochondrial number were reduced in the SN of aged male mice of both genotypes relative to their corresponding young control ($P < 0.01$, Figures 6(a)–6(c)). KO-aged male mice showed much lower CS activity mtDNA copy number and mitochondrial number in the SN than WT-aged male mice. There is no significant difference in CS activity, mtDNA copy number, and mitochondrial number in the SN between KO-young male mice and WT-young male mice. Supplementation with TP increased CS activity, mtDNA copy number, and mitochondrial number in the SN of WT-aged male mice ($P < 0.01$). Increased CS activity, mtDNA copy number, and mitochondrial number by TP were not found in KO-aged-TP mice. In addition, there were striking differences in the mitochondrial ultrastructure among the experimental groups. Compared with mitochondria from the SN of WT-young mice, which presented a normal mitochondrial structure with clear cristae, most mitochondria from the WT-aged or KO-aged male mice showed disorganized cristae. Supplementation with TP to WT-aged male mice improved the ultrastructural alterations of mitochondrial cristae in the SN, which was not observed in KO-aged male mice (Figure 6(d)). TP supplementation increased mitochondrial content in the SN of WT-aged male mice, but not in KO-aged male mice.

3.7. Nrf2 Deficiency Attenuated Testosterone Efficiency in Regulating Levels of Drp1, Mfn1, and OPA1 in the SN of Aged Male Mice. Mitochondrial dynamics take part in maintaining mitochondrial function, and abnormal mitochondrial dynamics are shown in aging and age-related neurodegenerative conditions. Therefore, we analyzed the alterations in the levels of Drp1 and its phosphorylation (pDrp1-S616), Mfn1, and two OPA1 forms (long OPA1: L-OPA1 and short OPA1: S-OPA1) in the SN of experimental mice, which are involved in regulating mitochondrial dynamics. Aged male mice of both genotypes showed reduced mRNA levels of *Drp1*, *Mfn1*, and *OPA1* in the SN compared with respective young control ($P < 0.01$, Figures 7(a)–7(c)). Nrf2 KO further reduced their mRNA levels in the SN of aged male mice. There was no significant difference between WT-young and KO-young mice in mRNA levels of *Drp1*, *Mfn1*, and *OPA1* in the SN. Supplementation with TP increased *Drp1*, *Mfn1*, and *OPA1* mRNA levels in the SN of WT-aged male mice. Increased mRNA levels of *Drp1*, *Mfn1*, and *OPA1* by TP were not detected in the SN of KO-aged-TP mice. Immunoblotting data showed the levels of Drp1, pDrp1-S616, Mfn1, L-OPA1, and S-OPA1 were significantly reduced in the SN of WT-aged mice relative to WT-young mice (Figures 7(d)–7(i)). They were much lower in KO-aged mice

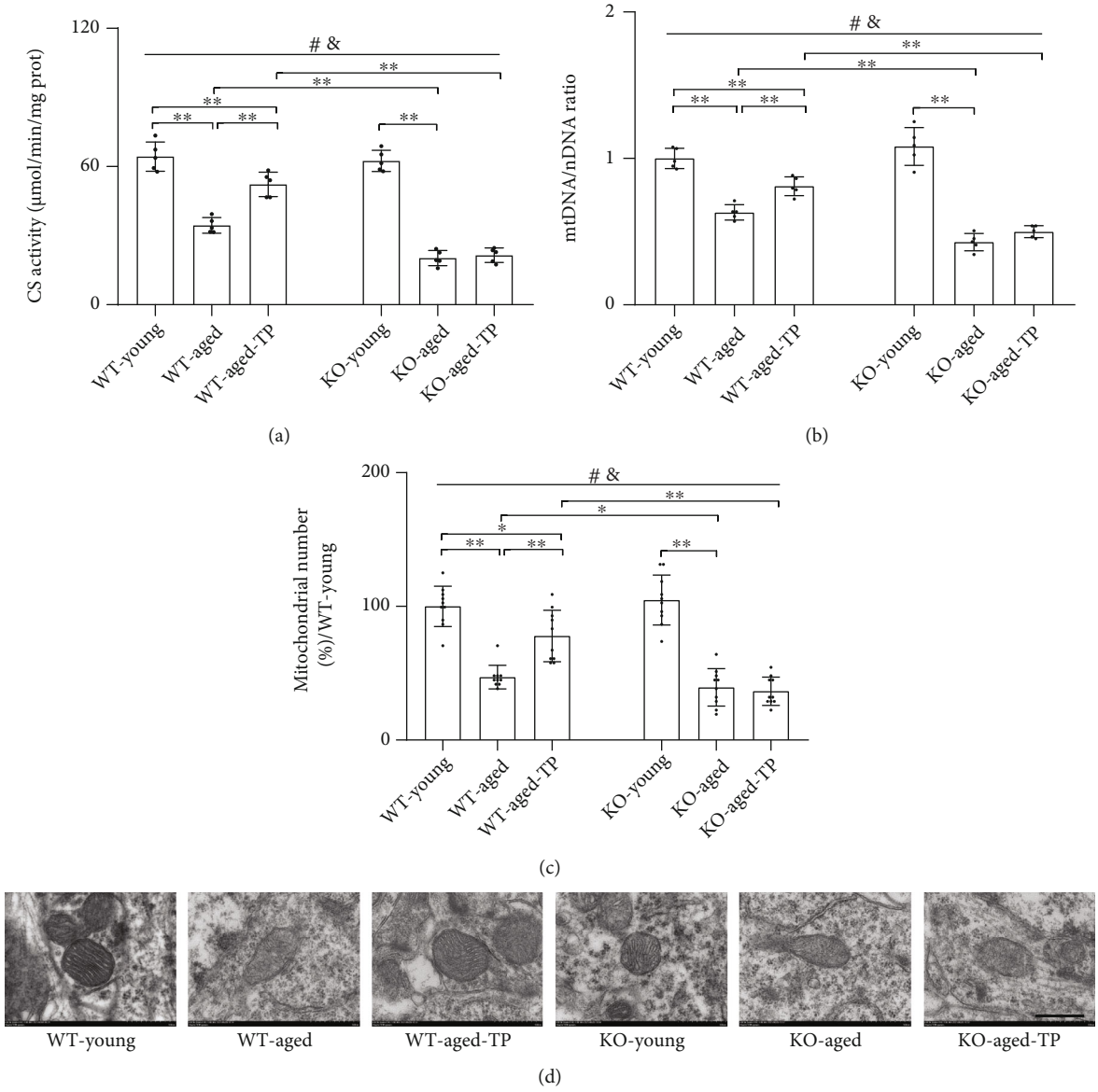
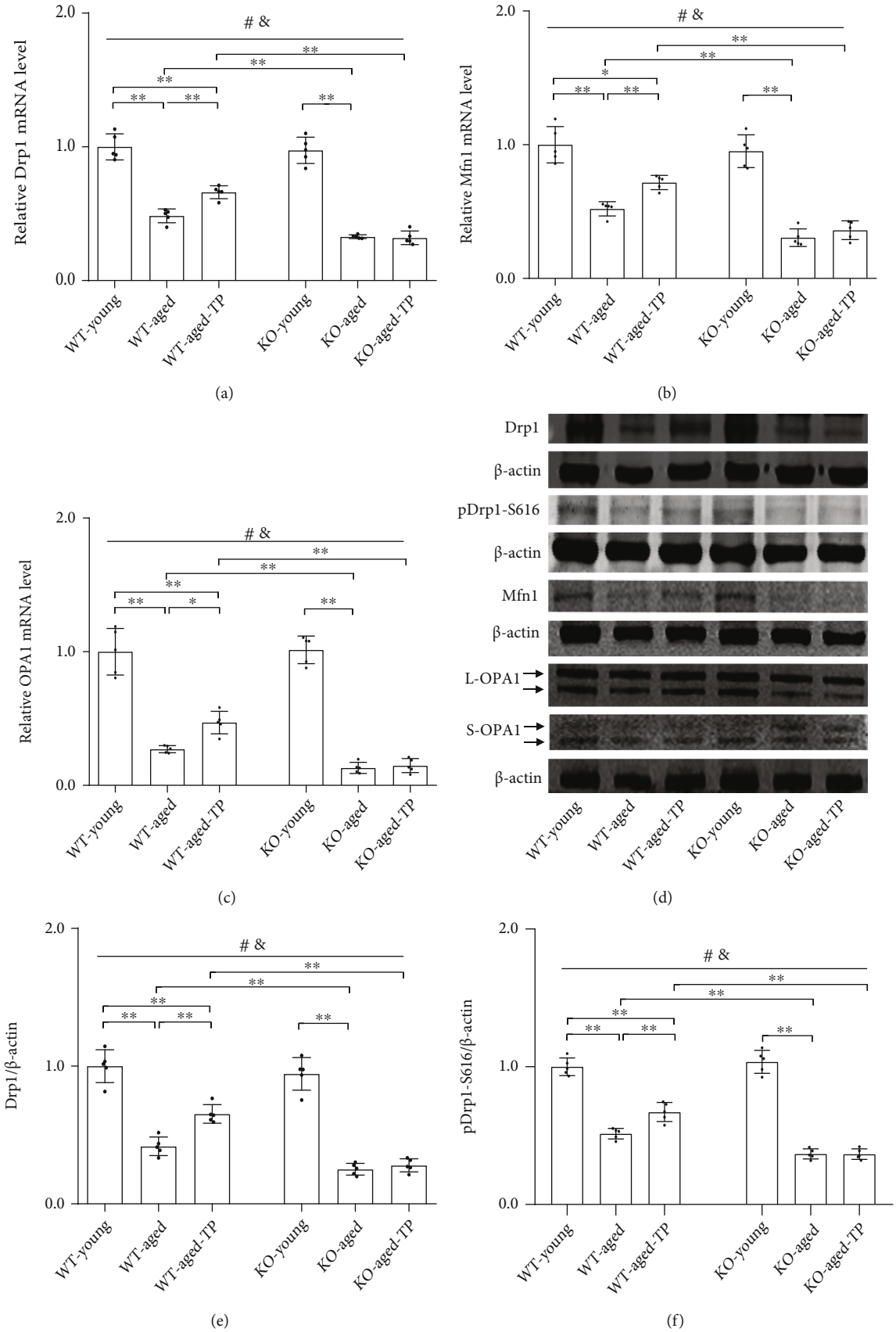


Figure 6: Effects of TP supplementation on mitochondrial content in the substantia nigra of Nrf2 KO-aged male mice. (a) CS activity was assessed by spectrophotometry. (b) mtDNA/nDNA was detected by qPCR. (c) Mitochondrial number was counted using Image-Pro Plus 6.0 by an electron microscope. (d) Mitochondrial ultrastructure images were taken by an electron microscope. Data were presented as mean ± SD; $n = 5$ for CS activity and mtDNA/nDNA; $n = 2$ for an electron microscope (mitochondrial number from an analysis of 5 images per sample from each group). Scale bar = 500 nm. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

than in WT-aged mice, except for S-OPA1 (Figure 7(i)). Significantly increased levels of Drp1, pDrp1-S616, Mfn1, L-OPA1, and S-OPA1 were present in the SN of WT-aged-TP mice compared with WT-aged mice and were not found in KO-aged-TP mice relative to KO-aged mice. TP supplementation increased the levels of Drp1, pDrp1-S616, Mfn1, and two OPA1 forms in the SN of WT-aged male mice, but not in Nrf2 KO-aged male mice.

3.8. The Effects of TP Supplementation on Nrf2 in the SN of Aged Male Mice. To explore whether testosterone induces Nrf2 expression or promotes its nuclear translocation, Nrf2 and its downstream target HO-1 were detected by immunoblotting. Relative to WT-aged mice, significantly increased Nrf2 levels in the SN as well as in nucleus fraction were detected in WT-aged-TP mice (Figures 8(a), 8(b), 8(d), and 8(e)). Elevated HO-1 levels were shown in the SN of WT-aged-TP mice compared with WT-aged mice ($P < 0.01$), and increased HO-1 levels in the SN were not detected in KO-aged-TP mice relative to KO-aged mice. Supplementation with TP increased Nrf2 levels and promoted its nuclear translocation in WT-aged male mice.

3.9. The Effects of Nrf2 Deficiency on the Testosterone Efficiency in GDX Young Male Mice. Under the condition of ruling out aging factor, the above parameters detected



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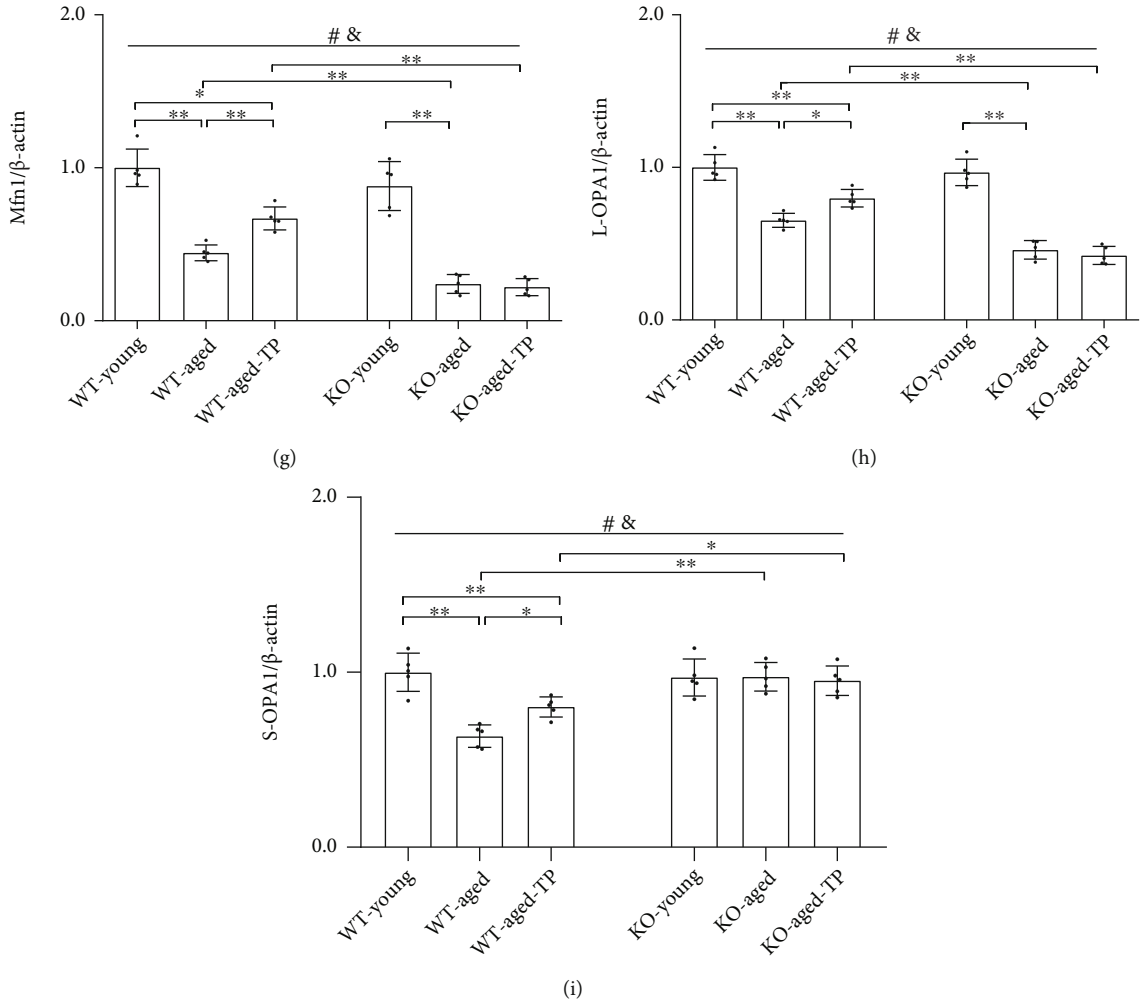


Figure 7: Effects of TP supplementation on mitochondrial dynamics in the substantia nigra of Nrf2 KO-aged male mice. (a) *Drp1*, (b) *Mfn1*, and (c) *OPA1* mRNA were revealed by qPCR. (d, e) Drp1, (d, f) pDrp1-S616, (d, g) Mfn1, (d, h) L-OPA1, and (d, i) S-OPA1 proteins were detected by immunoblotting. Data were presented as mean ± SD; $n = 5$. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

in Experiment 1 were analyzed in the young experimental animal models of Experiment 2 (Figures 9–15). Orchiectomy to young mice of both genotypes weakened open-field activity ($P < 0.01$, Figures 9(a)–9(e)). Gonadectomized young male mice of both genotypes showed the decreased nigrostriatal dopaminergic activity ($P < 0.01$, Figures 10(a)–10(f)), the increased oxidative stress (GSH/GSSG, MDA, mitochondrial H_2O_2, and mitochondrial 3-NT, $P < 0.01$, Figures 11(a)–11(e). 3-NT: AOD, $P < 0.05$; number, $P < 0.01$, Figures 11(f)–11(h)), the reduced mitochondrial function (MMP: WT, $P < 0.05$; KO, $P < 0.01$, Figure 12(a). Mitochondrial ATP, mitochondrial complexes I, IV, and V, $P < 0.01$, Figures 12(b), 12(c), 12(f), and 12(g)), and the decreased expression levels of PGC-1α, NRF-1, NRF-2, and TFAM ($P < 0.01$, Figures 13(a)–13(l)), as well as the lowered mitochondrial content (CS, mtDNA/nDNA, $P < 0.01$, Figures 14(a) and 14(b)) in the studied brain region. The increased Drp1 and pDrp1-S616, as well as the reduced Mfn1, L-OPA1, and S-OPA1 levels, were found in the SN of gonadectomized young male mice of both genotypes ($P < 0.01$, Figures 15(a)–15(i)). KO-GDX mice presented the aggravated effects of orchiectomy on the above parameters compared with WT-GDX mice, except for climbing (Figure 9(c)), 3-NT (AOD and number of 3-NT-ir cells, Figures 11(f)–11(h)), ATP (Figure 12(b)), PGC-1α (Figures 13(a), 13(e), and 13(i)), NRF-1 (Figures 13(b), 13(f), and 13(j)), TFAM (Figures 13(d), 13(h), and 13(l)), Mfn1 (Figures 15(b), 15(d), and 15(g)), OPA1 mRNA (Figure 15(c)), L-OPA1 (Figures 15(d) and 15(h)), and S-OPA1 (Figures 15(d) an(d) 15(i)). Supplementation with TP restored the observed parameters above in WT-GDX mice to WT-sham level except for DOPAC ($P < 0.05$, Figure 10(e)). The above parameters in KO-GDX-TP mice were ameliorated except for HVA (Figure 10(f)) and 3-NT (AOD and number of 3-NT-ir cells, Figures 11(f)–11(h)) relative to KO-GDX mice, but not reversed to KO-sham level except for NRF-2 (Figures 13(c), 13(g), and 13(k)). NRF-2 level in the SN of KO-GDX-TP mice reached the level of KO-sham mice.

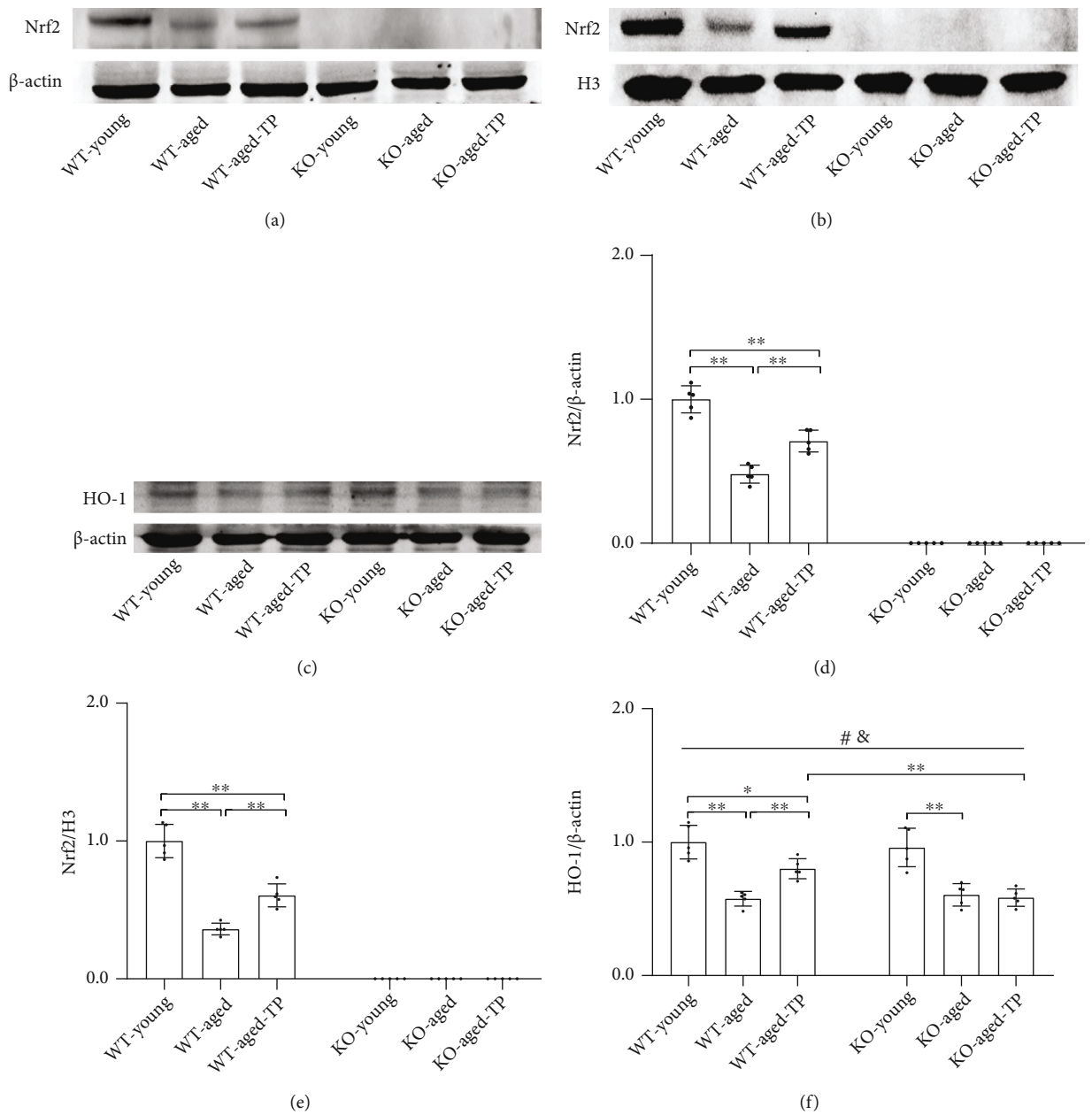
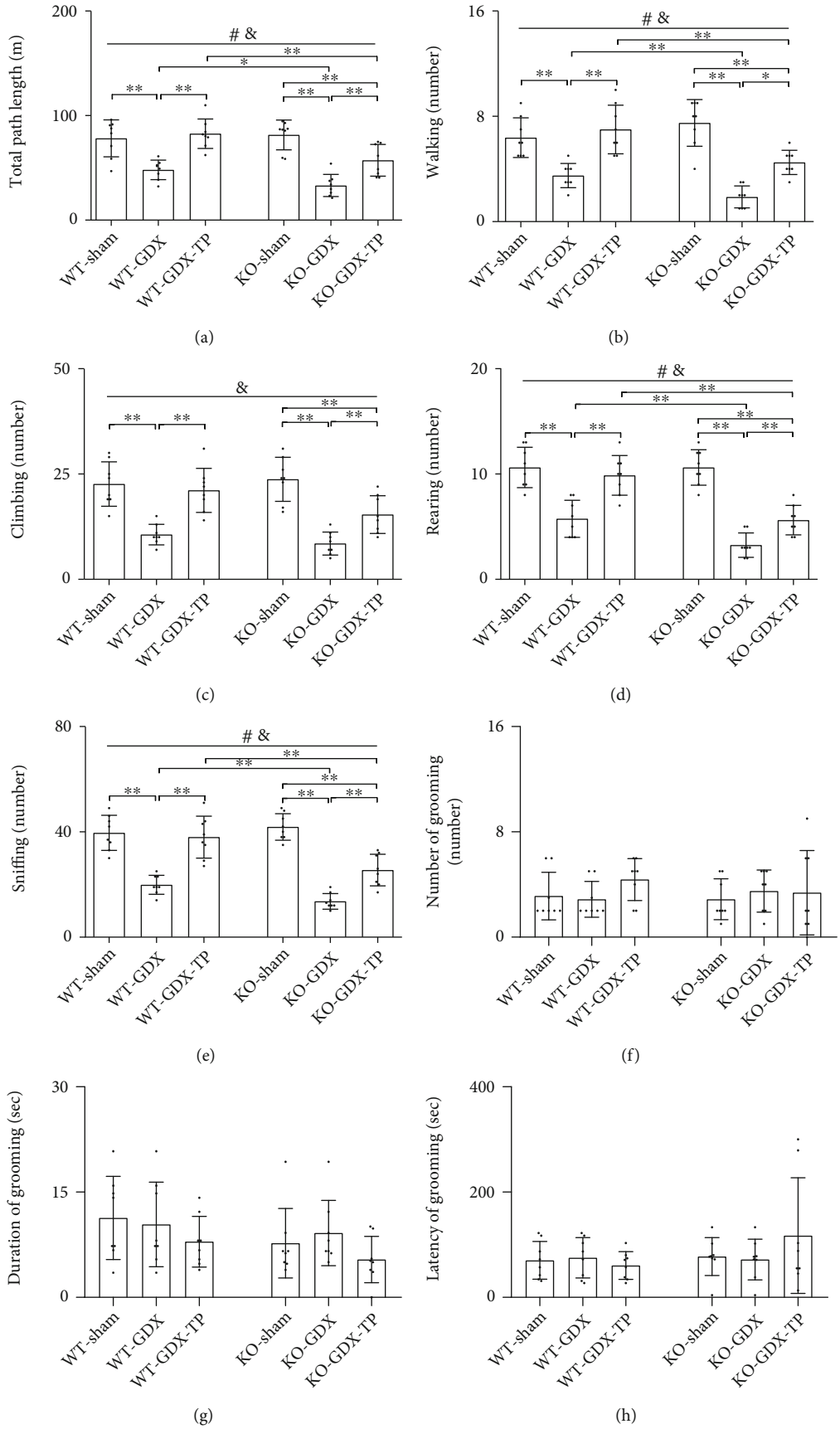


Figure 8: Effects of TP supplementation on Nrf2 in the substantia nigra of aged male mice. (a, d) Nrf2 in the SN, (b, e) Nrf2 in nucleus fraction, and (c, f) HO-1 protein in the SN were detected by immunoblotting. Data were presented as mean ± SD; $n = 5$. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

In this study, we demonstrated that WT-aged male mice presented uncoordinated walking gait, as well as declined open-field activity, nigrostriatal dopaminergic activity, oxidative balance, and mitochondrial function, as well as downregulated mitochondrial biogenesis and mitochondrial dynamics. Nrf2 deficiency exacerbated the deficits of the above parameters in aged male mice. Supplementation with testosterone to WT-aged male mice significantly ameliorated open-field activity, walking gait, nigrostriatal dopaminergic activity, oxidative balance, and mitochondrial function and upregulated mitochondrial biogenesis and mitochondrial dynamics. However, the above effects of testosterone on WT-aged male mice were not shown in Nrf2 KO-aged male mice. Orchidectomy to young male mice decreased mitochondrial function, downregulated mitochondrial biogenesis, and altered mitochondrial dynamics balance. Supplementation with testosterone to Nrf2 KO-GDX mice only ameliorated the alterations above but did not reverse them to sham level. Thus, Nrf2 plays an important role in ameliorating open-field activity, walking gait, and nigrostriatal dopaminergic activity of aged male mice by testosterone supplementation, which is related to the activated Nrf2 by TP. Nrf2 deficiency attenuated the efficiency of testosterone supplementation in improving



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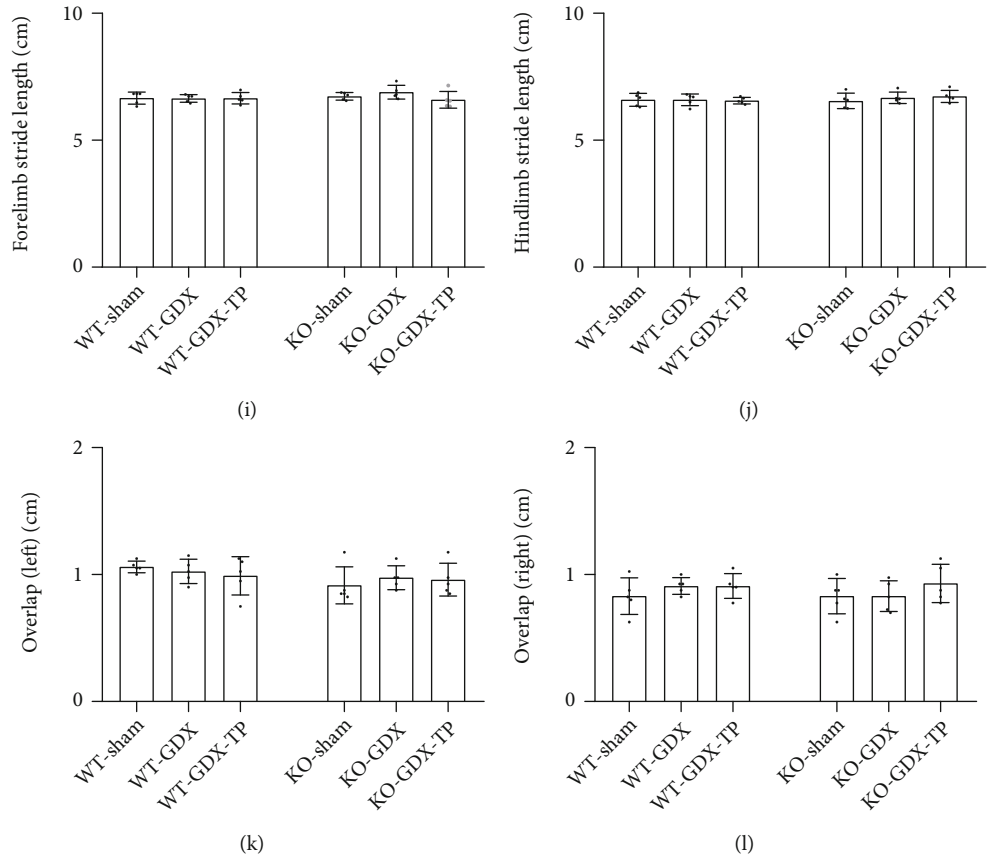


Figure 9: Effects of TP supplementation on open-field activity and walking gait of Nrf2 KO GDX male mice: (a) total path length, (b) walking, (c) climbing, (d) rearing, (e) sniffing, (f) number of grooming, (g) duration of grooming, (h) latency of grooming, (i) forelimb stride length, (j) hindlimb stride length, (k) overlap of left footprints, and (l) overlap of right footprints. Data were presented as mean ± SD; $n = 8$ for open-field test; $n = 5$ for footprint test. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

mitochondrial function of the substantia nigra in aged male mice via mitochondrial biogenesis and mitochondrial dynamics to some extent.

Motor activity gradually declines during the aging process [29], and this decline is related to the age-related decline of nigral neuronal function [29, 31]. A persistent loss of nigrostriatal neurons results in severe locomotor impairment, such as altered gait pattern [32]. Previous studies demonstrated that androgen influences brain function to induce behavioral alterations [14, 33]. Androgen supplementation increases motor and exploratory behaviors of aged male rodents [14, 18, 33] and ameliorated motor symptoms of men with PD to some extent [34]. The amelioration of motor and exploratory behaviors in testosterone-supplemented aged animals is related to nigrostriatal dopaminergic activity enhanced by androgen [14, 15]. The present study showed that supplementation with testosterone significantly improved motor and exploratory behaviors, as well as walking gait of WT-aged male mice and enhanced nigrostriatal dopaminergic activity. However, the above effects of testosterone supplementation on WT-aged male mice were not found in Nrf2 KO-aged male mice. The administration of testosterone to Nrf2 KO-aged male mice neither improved motor and exploratory behaviors as well as walking gait nor enhanced nigrostriatal dopaminergic activity, which demonstrated that Nrf2 might be required for testosterone to ameliorate their neurochemical defects of the nigrostriatal dopaminergic system in aged male mice. Nrf2 is widely expressed in the central nervous system including the SN. As a master transcription factor, Nrf2 binds ARE in the promoters of Nrf2 target genes [9, 35]. When it is activated, a series of cytoprotective enzymes and antioxidants are induced [35], such as HO-1. Nrf2 with its downstream gene products constitutes the main antioxidant defense system to degrade free radicals [9, 35]. The previous studies showed that Nrf2 protects nigrostriatal dopaminergic neurons and maintains their normal activity [9]. In PD, remaining dopaminergic neurons exhibit stronger nuclear Nrf2 immunoreactive staining [36]. Thus, our results above indicated that Nrf2 might determine the ameliorative effects of testosterone supplementation on the nigrostriatal dopaminergic system of aged male mice.

How does testosterone exerts its ameliorative effects on the nigrostriatal dopaminergic system of aged male animals has not been elucidated. Improved mitochondrial function might explain the ameliorative effects of TP supplementation on the nigrostriatal dopaminergic system during aging process by modulating oxidative stress, mitochondrial

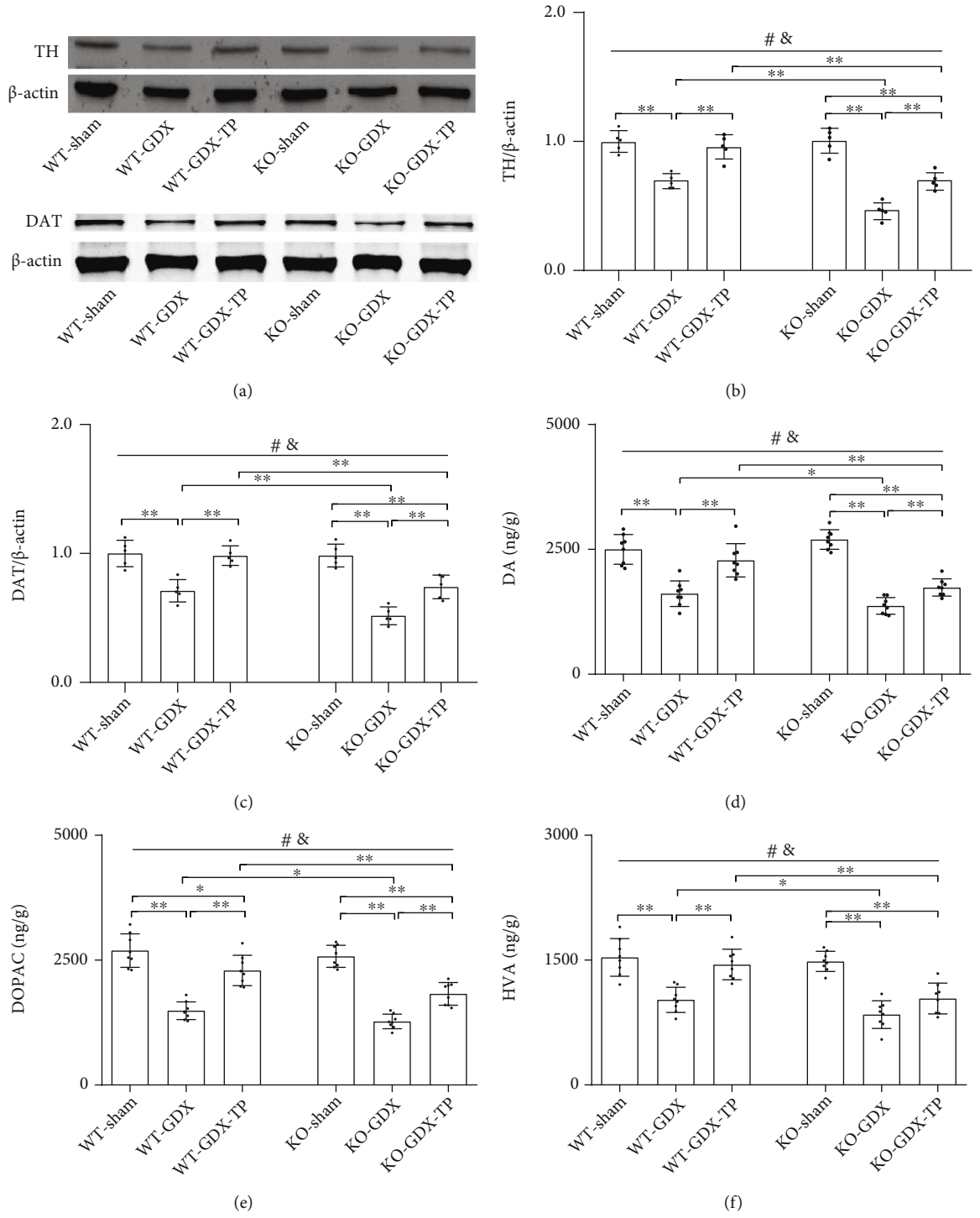
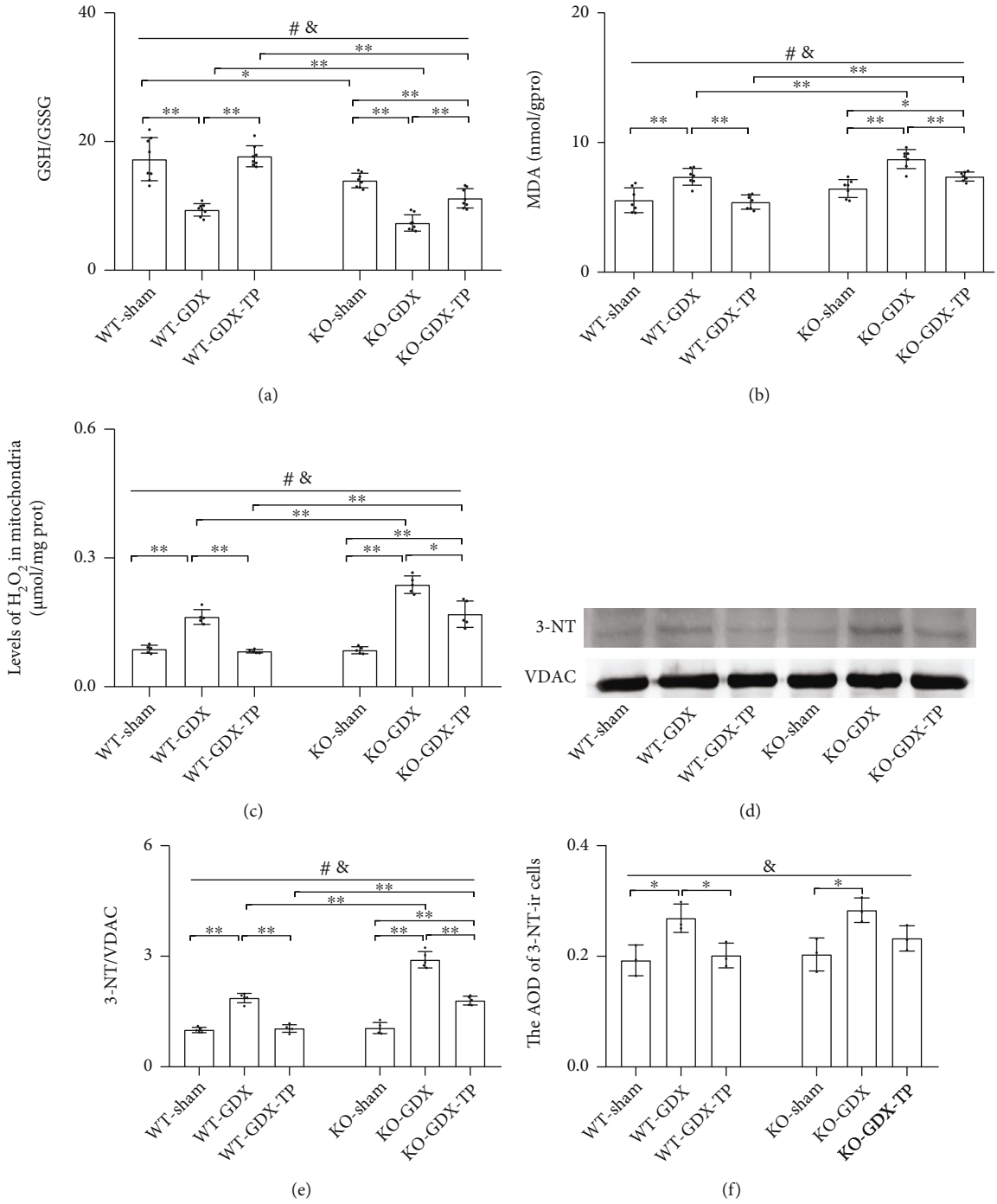


FIGURE 10: Effects of TP supplementation on dopaminergic activity in the caudate putamen of Nrf2 KO GDX male mice. (a, b) TH and (a, c) DAT were detected by immunoblotting. (d) DA, (e) DOPAC, and (f) HVA were measured by LC-MS/MS assay. Data were presented as mean ± SD; $n = 5$ for TH and DAT; $n = 8$ for DA, DOPAC, and HVA. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

biogenesis, and mitochondrial dynamics. Oxidative stress is characterized by an unbalance between overproduced ROS and antioxidant defenses in cells. It is one of the major factors in aging and in aging-related neurodegenerative diseases [37]. Under physiological conditions, cellular ROS are eradicated by the endogenous antioxidative defense systems. However, under pathological circumstances, mitochondrial dysfunction, such as reduced MMP (an important indicator of mitochondrial function) and impaired oxidative phosphorylation, produces excessive ROS, resulting in oxidative damage to cells by targeting proteins, lipids, or DNA [2]. The mitochondrion is a main organelle producing



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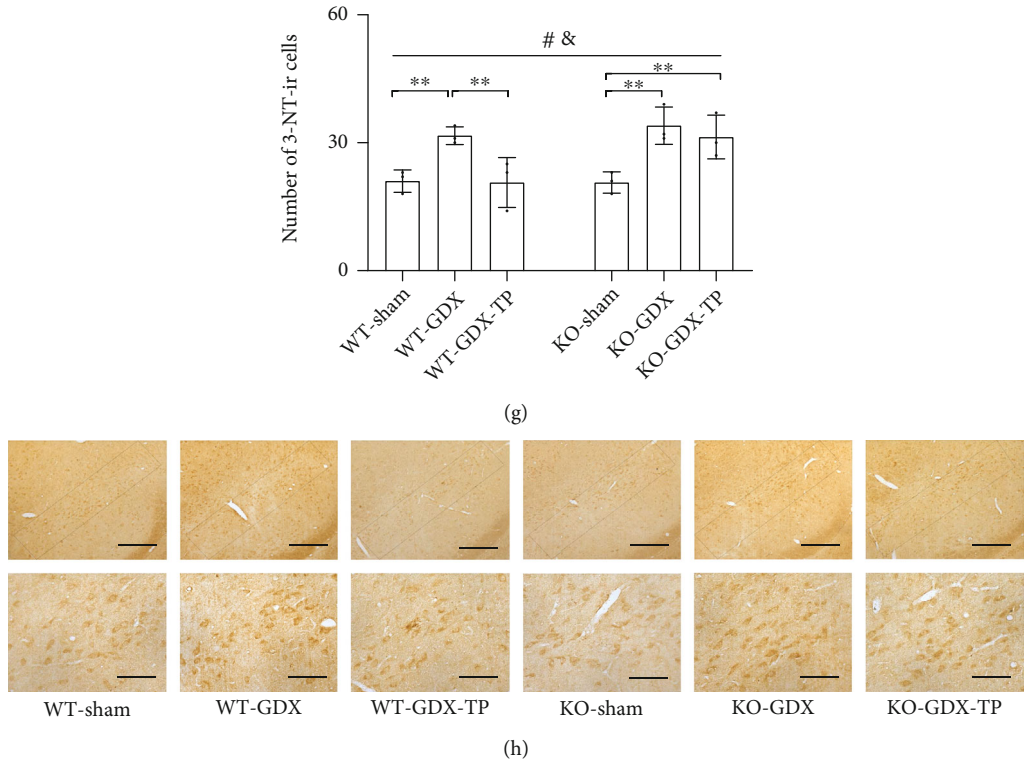
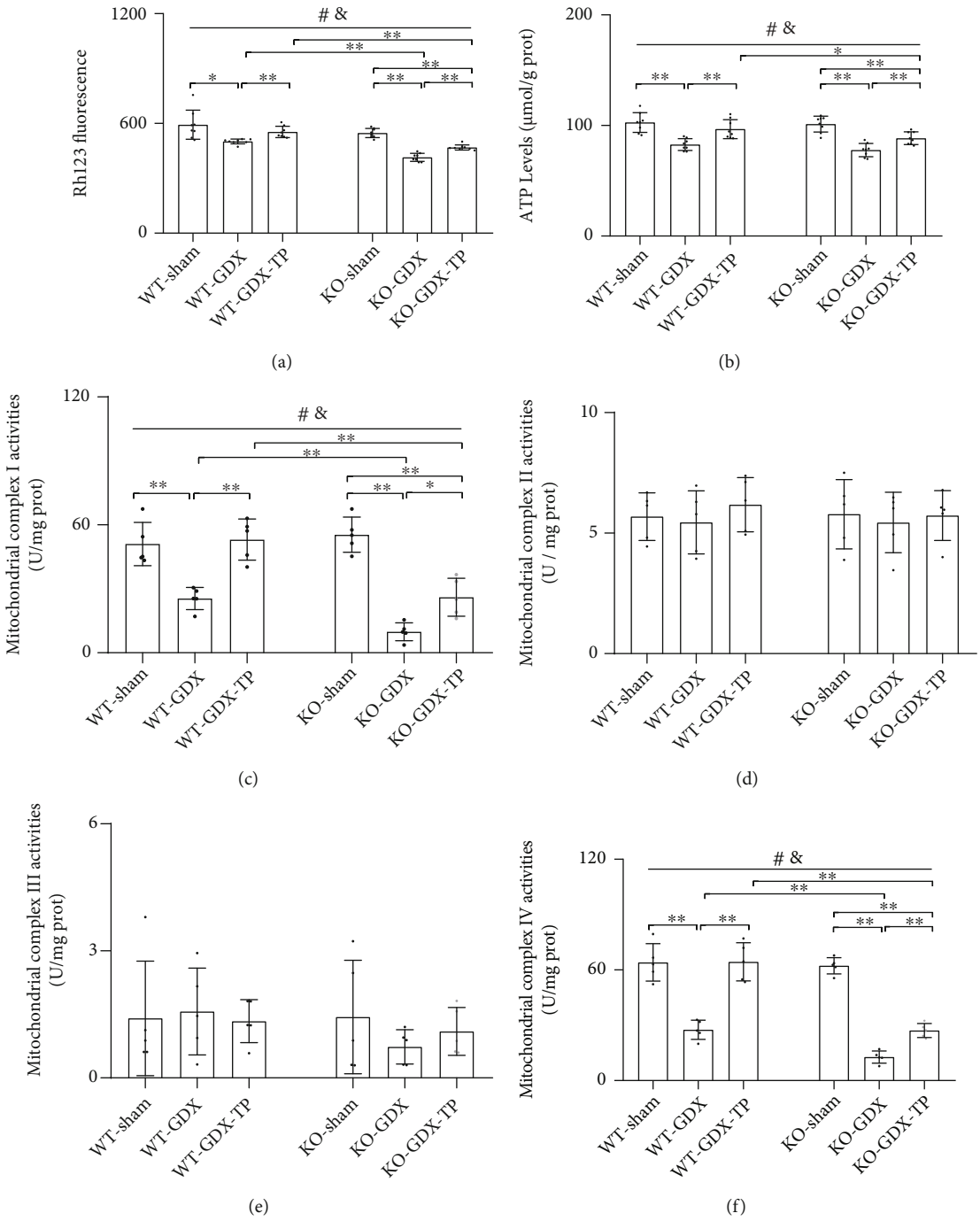


Figure 11: Effects of TP supplementation on oxidative balance in the substantia nigra of Nrf2 KO GDX male mice. (a) GSH/GSSG, (b) MDA, and (c) mitochondrial H_2O_2 were assessed by spectrophotometry. (d, e) Mitochondrial 3-NT was measured by immunoblotting; (f–h) 3-NT in the SN was detected by immunohistochemistry. Data were presented as mean ± SD; $n = 8$ for GSH/GSSG; $n = 7$ for MDA; $n = 5$ for mitochondrial 3-NT; $n = 3$ for 3-NT immunohistochemistry. Scale bars = 50 μm (lower panel); scale bars = 200 μm (upper panel). $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

superoxide anion (O_2^-). It is involved in oxidative damage to macromolecules and can be converted to O_2 and H_2O_2. GSH and GSSG are two different forms of glutathione. Glutathione peroxidase catalyzes the reduction of H_2O_2 via GSH to produce GSSG and H_2O. MDA is an important indicator for detecting lipid peroxidation of biological membranes. It is the peroxidation product of phospholipids or lipoproteins of cytoplasmic membranes [38]. 3-NT is another biomarker of oxidative stress to predict the level of oxidative damage [39, 40]. It is formed due to nitration of protein-bound and free tyrosine residues by reactive peroxynitrite molecules. The previous study showed that the levels of Nrf2 and its downstream gene products, such as NQO-1 and HO-1, are higher in TP-treated aged male rats than in their counterpart control [15]. TP-treated aged male rats show decreased MDA and GSH/GSSG in the SN compared with control [15, 18]. In the present study, decreased GSH/GSSG and increased MDA as well as mitochondrial H_2O_2 and 3-NT revealed the existed oxidative damage in the SN of aged male mice, especially in Nrf2 KO-aged male mice. Decreased MMP and mitochondrial ATP levels, as well as activities of mitochondrial complexes I, IV, and V, indicated mitochondrial dysfunction in the SN of aged male mice. Testosterone supplementation decreased MDA, as well as mitochondrial H_2O_2 and 3-NT levels, and increased GSH/GSSG ratio, MMP, mitochondrial ATP content, and activities of mitochondrial complexes I, IV, and V in the SN of WT-aged male mice. The results above showed improved mitochondrial function in WT TP-treated aged male mice. By comparing the results of 3-NT immunohistochemistry with those of 3-NT immunoblotting, it was found that although both methods detected a significant increase in 3-NT levels in the SN of WT-aged male mice before TP treatment, immunohistochemistry-revealed 3-NT levels only showed decreased trend in WT-aged-TP mice, not reaching significance. However, immunoblotting detected significantly decreased 3-NT levels in WT-aged-TP mice. The former observed the cells in the SN, while the latter located isolated mitochondria from the SN, which will more accurately reflect the subtle changes in damaged organelles. Mitochondria, as primary sources of ROS, were damaged in WT-aged male mice. The mitochondrial ultrastructure by an electron microscope supported the finding above. WT-aged mice showed ultrastructural abnormalities of mitochondria in a way, such as unclear cristae, which was improved by TP supplementation. However, improved oxidative balance status and mitochondrial function were only shown in the SN of TP-treated WT-aged male mice, not in TP-treated Nrf2 KO-aged male mice. It was suggested that Nrf2 deficiency blocked the efficiency of testosterone replacement in ameliorating mitochondrial function in the SN of aged male mice, leading to oxidative damage to SN.

Aging and aging-related neurodegenerative disorders are often accompanied by mitochondrial dysfunction [21].



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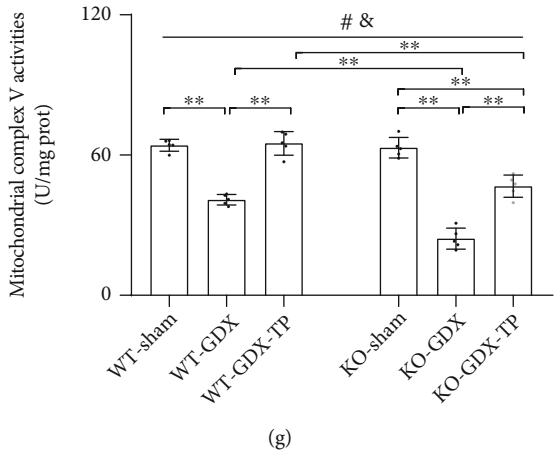
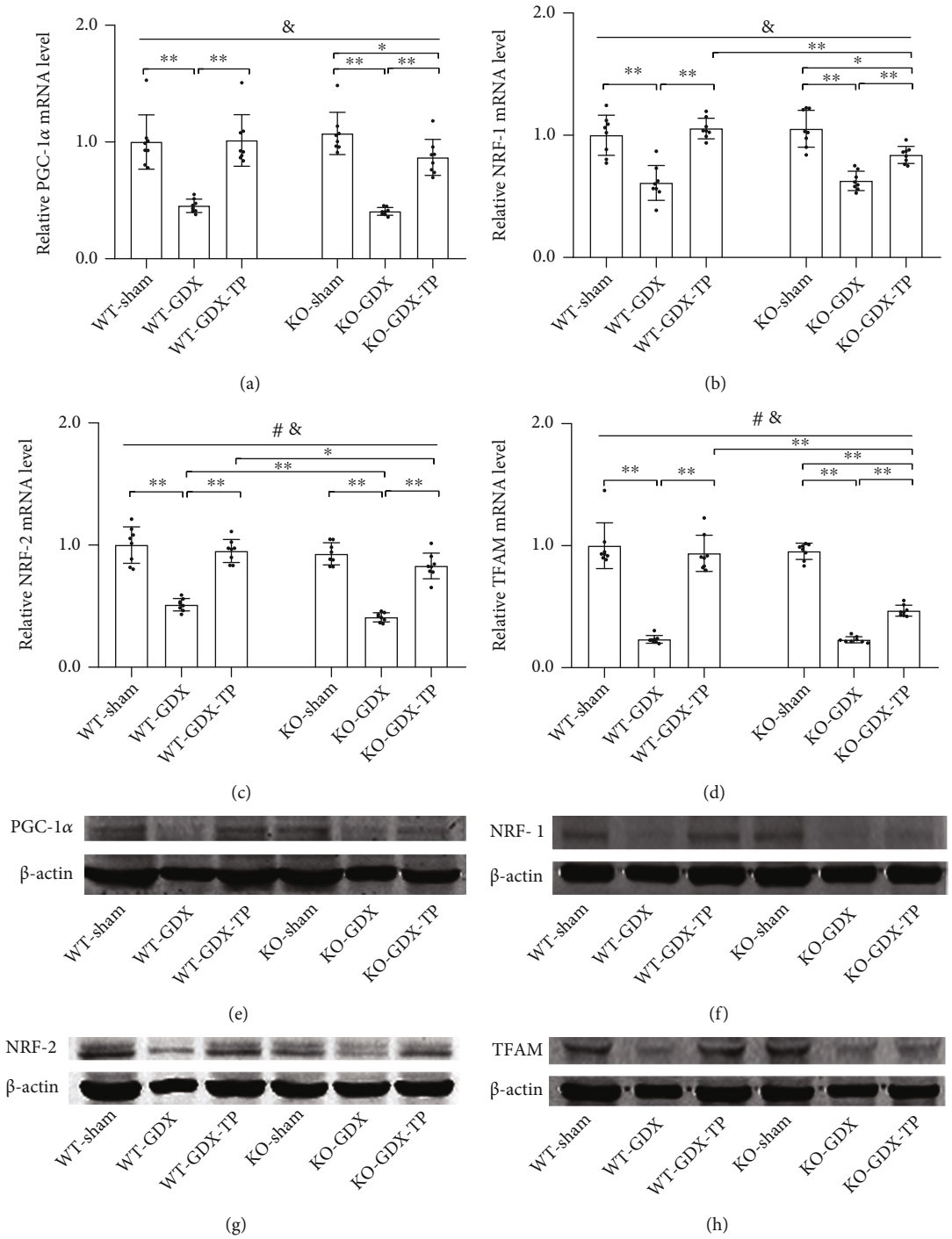


Figure 12: Effects of TP supplementation on mitochondrial function in the substantia nigra of Nrf2 KO GDX male mice. (a) Mitochondrial membrane potential was revealed using the Rh123 fluorescence method. (b) Mitochondrial ATP levels, (c) mitochondrial complex I activity, (d) mitochondrial complex II activity, (e) mitochondrial complex III activity, (f) mitochondrial complex IV activity, and (g) mitochondrial complex V activity were revealed by spectrophotometry. Data were presented as mean ± SD; $n = 8$ for MMP and mitochondrial ATP levels; $n = 5$ for mitochondrial complexes I-V. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

During aging process, promoting mitochondrial biogenesis might be a cellular strategy to maintain normal mitochondrial function [6]. Mitochondrial biogenesis is mainly regulated by PGC-1α, a key regulator of mitochondrial biogenesis and antioxidative defense [8, 41]. It controls the rate of the mitochondrial biogenesis through its downstream targets, NRF-1, NRF-2, and TFAM [41, 42]. *In vitro* study showed that testosterone increases PGC-1α, NRF-1, and TFAM at transcription level in C_2C_{12} myotubes [43, 44] and flutamide (androgen receptor antagonist) reduces the testosterone-induced upregulation of PGC-1α [44], NRF-1, and TFAM [43]. Moreover, testosterone deficiency caused by orchiectomy decreases PGC-1α, NRF-1, and TFAM gene expression in the adult rat hippocampus and supplementation of testosterone to orchiectomy adult rats restores them in the hippocampus to control levels [19]. Similarly, we found in the present study that orchiectomy reduced PGC-1α, NRF-1, NRF-2, and TFAM gene expression in the SN of WT-young male mice. Reduced PGC-1α, NRF-1, NRF-2, and TFAM gene expression, with decreased CS activity and mtDNA copy number in the SN of GDX WT-young male mice, was reversed to sham levels by TP supplementation, which suggested that mitochondrial biogenesis in the SN is androgen-related. Mitochondrial biogenesis has been found to decline in the skeletal muscle and in the brain during aging [18, 45]. During the aging process, PGC-1α expression level declines in the skeletal muscle as well as in the brain and this can be reversed in the skeletal muscles by exercise training of aged male rats [46] and ameliorated in the brain by TP supplementation to aged male rats [18], which demonstrated the aging skeletal muscle cells and neurons still remain the ability to upregulate mitochondrial biogenesis through increasing PGC-1α expression [18, 46]. Narasimhan et al. found that the PGC-1α protein levels were decreased in the skeletal muscle of Nrf2 KO-aged mice compared with WT-aged mice [47]. In the present study, we found that PGC-1α and its downstream target expression levels in the SN were significantly reduced in WT-aged mice and Nrf2 KO further decreased their expression levels in the SN upon aging. In addition, CS activity, mtDNA copy number, and mitochondrial number were significantly reduced in the SN of KO-aged mice due to Nrf2 deficiency. mtDNA copy number is frequently used as an index of mitochondrial content. CS activity is a more accurate indicator for mitochondrial content in tissues than mtDNA copy number [48]. Mitochondrial number in cells by electron microscopy directly displays the status of mitochondrial biogenesis and mitochondrial content. Supplementation with TP to WT-aged mice increased expression of PGC-1α and its downstream targets, as well as CS activity, mtDNA copy number, and mitochondrial number in the SN, and such effects of TP supplementation on them in the SN were blocked in Nrf2 KO-aged mice due to Nrf2 deficiency. A previous *in vitro* study revealed that silencing the PGC-1α gene in SH-SY5Y cells results in the reduction of MMP and intracellular ATP content as well as the elevation of intracellular H_2O_2 generation [49]. Thus, the improvement of mitochondrial function (i.e., increased MMP, mitochondrial ATP, and complex activities, as well as decreased MDA, mitochondrial H_2O_2, and 3-NT and increased GSH/GSSG) in the SN of TP-treated WT-aged mice was associated with the TP-promoted mitochondrial biogenesis in large extent. Nrf2 played an important role in the effects of testosterone supplementation on the SN mitochondrial biogenesis of aged male mice.

The balanced mitochondrial dynamics is another important factor for improving mitochondrial function in the maintenance of normal cell activity [50–52]. Mitochondria undergo continuous rounds of fusion and fission in their life and form into dynamic networks within cells. Balanced mitochondrial dynamics are regulated by the GTPase family of proteins including Drp1, Mfn1/Mfn2, and OPA1 [53–55]. Drp1 is responsible for the mitochondrial fission [53]. Drp1 activity is regulated by its phosphorylation. Phosphorylation



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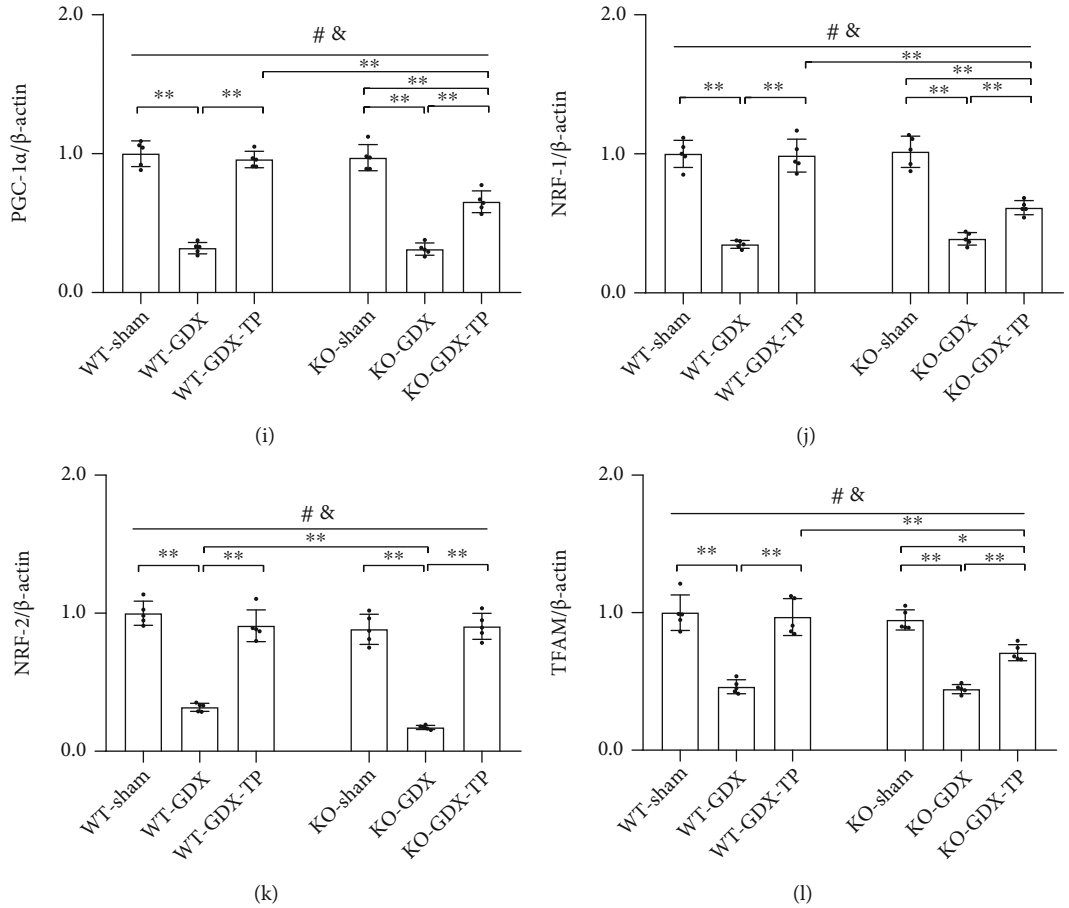


Figure 13: Effects of TP supplementation on mitochondrial biogenesis in the substantia nigra of Nrf2 KO GDX male mice. (a) *PGC-1α*, (b) *NRF-1*, (c) *NRF-2*, and (d) *TFAM* mRNA were revealed by qPCR. (e, i) PGC-1α, (f, j) NRF-1, (g, k) NRF-2, and (h, l) TFAM proteins were detected by immunoblotting. Data were presented as mean ± SD; $n = 8$ for qPCR; $n = 5$ for western blot. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

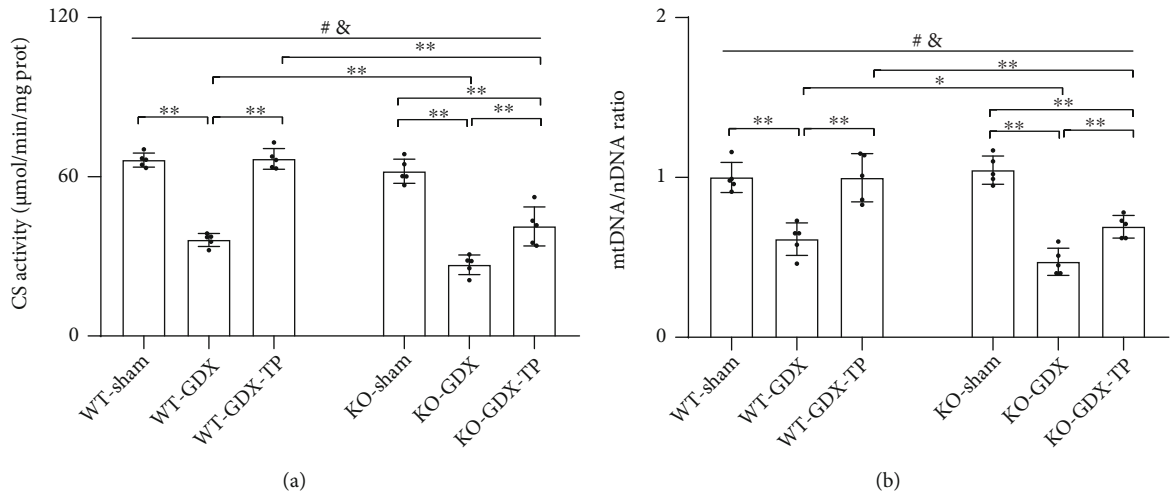
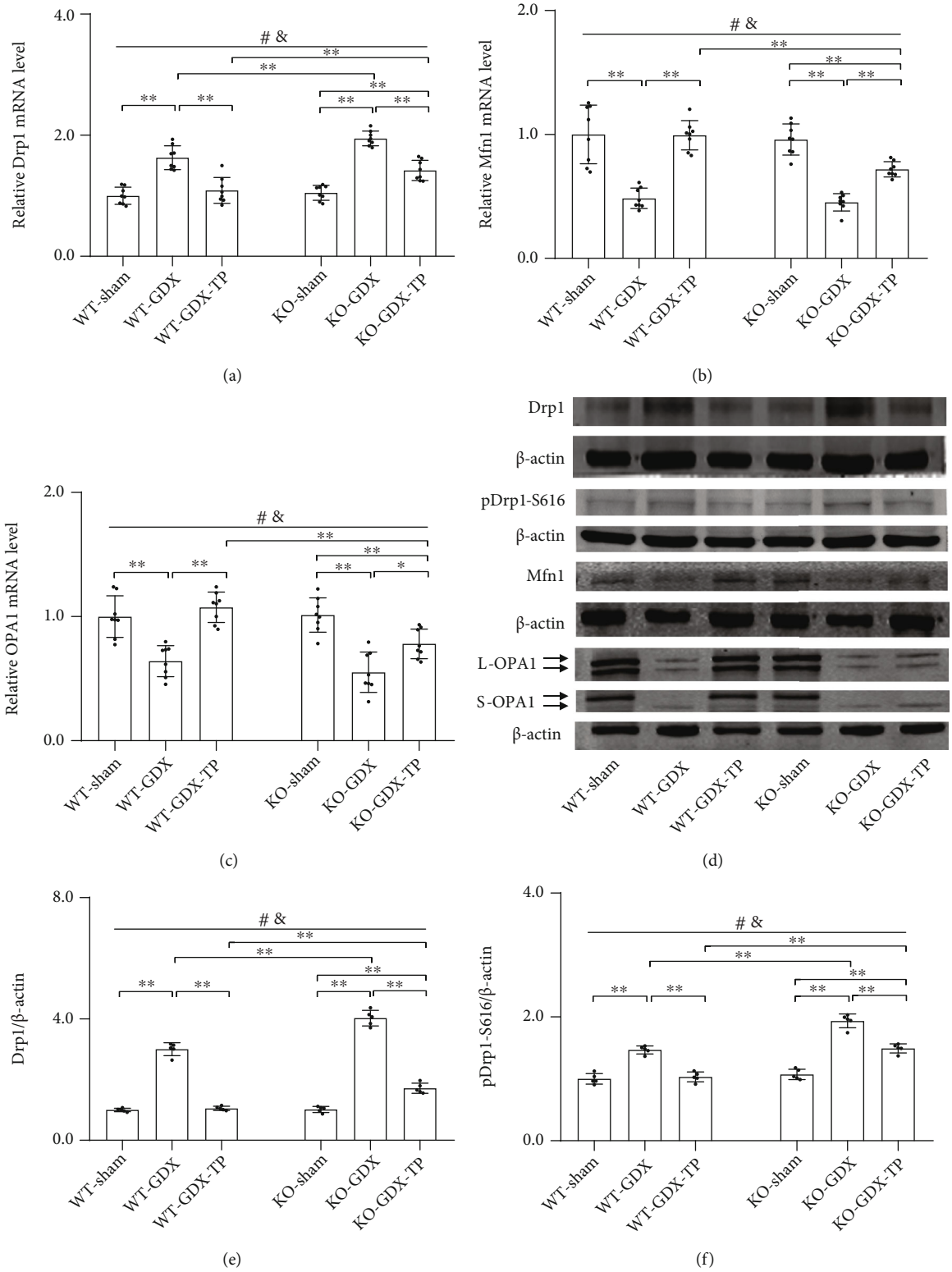


Figure 14: Effects of TP supplementation on mitochondrial content in the substantia nigra of Nrf2 KO GDX male mice. (a) CS activity was assessed by spectrophotometry. (b) mtDNA/nDNA was detected by qPCR. Data were presented as mean ± SD; $n = 5$. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.



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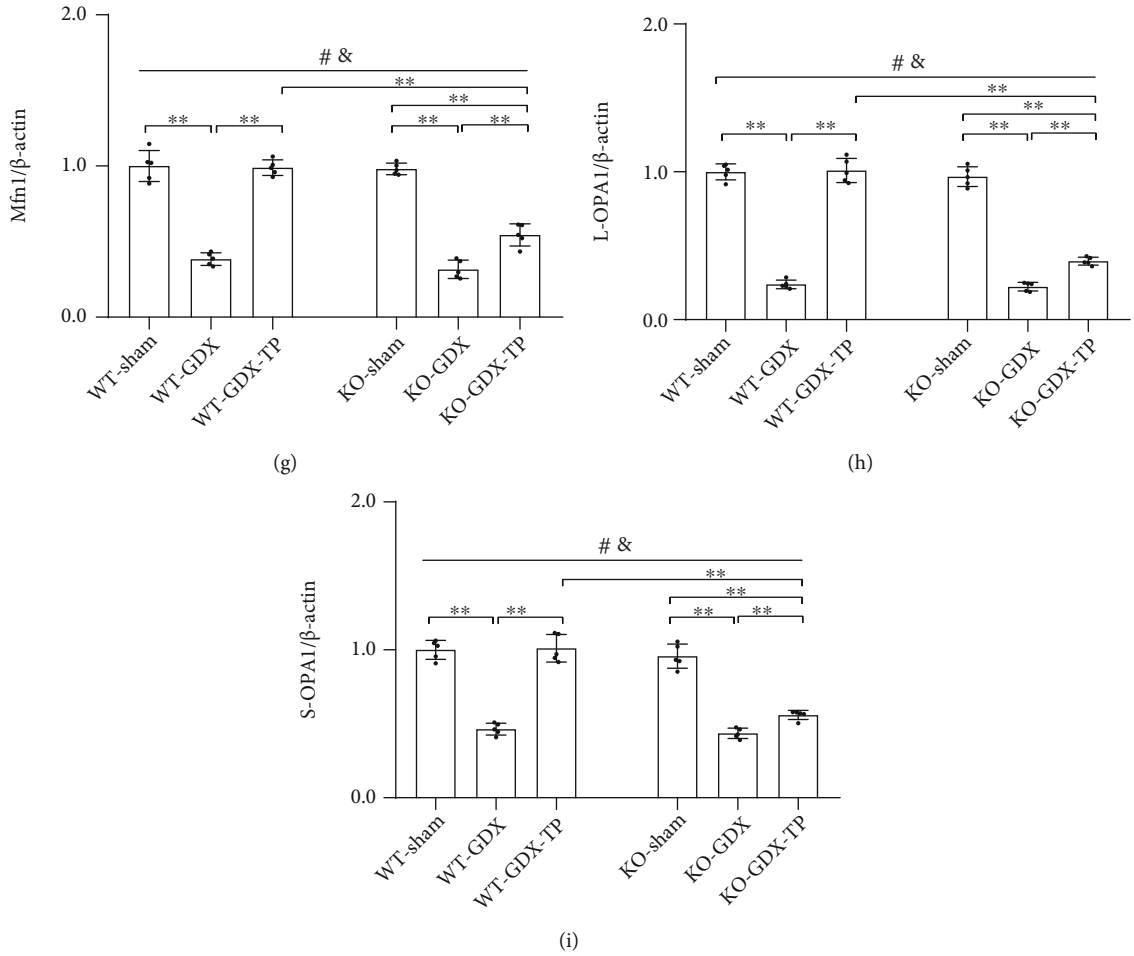

Figure 15: Effects of TP supplementation on mitochondrial dynamics in the substantia nigra of Nrf2 KO GDX male mice. (a) *Drp1*, (b) *Mfn1*, and (c) *OPA1* mRNA were revealed by qPCR. (d, e) Drp1, (d, f) pDrp1-S616, (d, g) Mfn1, (d, h) L-OPA1, and (d, i) S-OPA1 proteins were detected by immunoblotting. Data were presented as mean ± SD; $n = 8$ for qPCR; $n = 5$ for immunoblotting. $^{\#}P < 0.05$ main effect of genotype by two-way ANOVA; $^{\&}P < 0.05$ main effect of treatment by two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$.

at Ser616 enhances Drp1-mediated fission [56]. Mfn1/Mfn2 and OPA1 are involved in the mitochondrial fusion [54, 55]. OPA1 activity is regulated by L-OPA1 and S-OPA1 through OPA1 cleavage. Both L-OPA1 and S-OPA1 are necessary for mitochondrial fusion; however, L-OPA1 or S-OPA1 alone is not sufficient for fusion under normal conditions [57]. The expression of Drp1 and OPA1 seemed to be affected by androgen levels. In an androgenized rat model for polycystic ovary syndrome, androgen induces upregulation of Drp1 [58]. Treatment of androgen-sensitive prostate cancer cells with an androgen receptor agonist or antagonist revealed that Drp1 is transcriptionally regulated by them [59]. In addition, it was found that testosterone increases fusion protein OPA1 expression in C_2C_{12} cells [43]. In the present study, we found that testosterone deficiency induced by orchiectomy disrupted balanced mitochondrial dynamics in the SN of WT young male mice. Orchiectomy increased Drp1 and pDrp1-S616 levels and decreased Mfn1, L-OPA1, and S-OPA1 levels in the SN of WT young mice. It was indicated that there might be more fragmented mitochondria in the SN of GDX young male mice, which affects mitochondrial function and metabolism [51]. Unlike GDX young male mice, aged male mice showed the decreased levels of Drp1, pDrp1-S616, Mfn1, L-OPA1, and S-OPA1 in the SN. The difference related to mitochondrial dynamics between aged male mice and GDX young male mice showed that the ability of both mitochondrial fusion and mitochondrial fission was downregulated in aged male mice. Previous studies revealed the dysregulated mitochondrial dynamics in aging-related neurodegenerative diseases [60, 61]. Specifically, increased mitochondrial fission and reduced mitochondrial fusion are major features in aging-related neurodegenerative diseases [60, 62]. However, in contrast with the different expression state between fission proteins and fusion proteins in pathological conditions, natural aging shows the declined expression of both fission proteins and fusion proteins [63, 64]. Mitochondrial fission-fusion mRNA and protein expression levels are differentially altered in the aging process, but they reach significantly lower levels of mitochondrial dynamics proteins in older animals [64]. Decreased Drp1 was found in several aged mouse tissues including neurons, in cultured aged human endothelial cells, or in old human skeletal muscle [63–65], and reduced Mfn1 and OPA1 were also detected in the skeletal muscle of aged

mice [64, 65]. The balance between mitochondrial fusion and fission is clearly crucial for neuronal function [51]. Therefore, aged organisms might try to maintain a balanced mitochondrial dynamics through downregulating both fission and fusion protein levels. However, altered mitochondrial dynamics by downregulating mitochondrial dynamics proteins in the aging process might not be efficient enough to maintain normal mitochondrial structures and functions in aged animals [65, 66] as young animals do. Supplementation with TP upregulated Drp1 and pDrp1-S616 involved in fission, as well as Mfn1, L-OPA1, and S-OPA1 related to fusion in the SN of WT-aged male mice, thus ameliorating their mitochondrial dynamics in the SN. However, this effect of TP supplementation in WT-aged mice was not detected in Nrf2 KO-aged male mice. Nrf2 deficiency blocked efficiency of TP supplementation in aged male mice. The present results indicated that the regulation of mitochondrial dynamics in aged male mice was affected by testosterone levels and Nrf2 was a key factor for TP upregulation of mitochondrial dynamics in the SN of aged male mice. Mitochondrial fusion contributes to increasing mitochondrial ATP production [67]; therefore, the increased ATP levels, as well as improved status of nigrostriatal dopaminergic neurons in the SN of TP-treated WT-aged male mice, suggested that the final net effects of altered mitochondrial dynamics by TP supplementation might promote mitochondrial fusion, which should be testified in the future studies.

Both testosterone level declines and age-related mitochondrial dysfunction are present in aging men and rodents [18, 46, 68]. Coincidentally, Nrf2 also decreases during aging process [69]. It seemed that there was close association among them. An age-related decline in Nrf2 function might play an important role between loss of testosterone and declined mitochondrial function during aging process. It was found that Nrf2 is involved in the maintenance of mitochondrial function through modulating cellular redox balance and MMP [70]. Activation of Nrf2 would contribute to the improved mitochondrial function during aging process. In the present study, we found that supplementation with TP increased Nrf2, as well as its downstream target HO-1 expression, and promoted Nrf2 nuclear translocation in the aged male mice. Thus, loss of testosterone during aging process might reduce activation of Nrf2, affecting mitochondrial function. Ameliorated mitochondrial function in the SN of WT-aged-TP mice was related to Nrf2 activation by TP supplementation to large extent. Nrf2 deficiency exacerbated mitochondrial dysfunction in the SN of aging male mice, and TP supplementation did not ameliorate the exacerbated mitochondrial dysfunction in KO-aged male mice. The following might explain the difference of mitochondrial function in the SN of TP-supplemented aged male mice of both genotypes. One was direct involvement of Nrf2 in the regulation of mitochondrial function in this process. The study by Piantadosi et al. checked for Nrf2-binding sites in the NRF-1 promoter and found four AREs in the NRF-1 gene promoter [71]. Nrf2 binds to AREs of NRF-1 promoter, activates NRF-1, and induces mitochondrial biogenesis via TFAM [71]. TP supplementation to aged male mice increased nuclear Nrf2 levels in the SN, thus improving mitochondrial function of aged male mice through promoting mitochondrial biogenesis. This effect of TP supplementation was significantly blocked due to Nrf2 knockout. Another was the oxidative stress milieus of cells, whether it was ameliorated by the regulation of antioxidants and antioxidant gene expression. The oxidative stress status among experimental groups was different in the present study. Significantly increased oxidative stress was present in KO-aged mice, as well as KO-aged-TP mice, compared with WT-aged mice. Testosterone has protective effects in a low oxidative stress environment [22]. And these protective effects will be weakened or even harmful in the state of high oxidative stress [22, 72]. In addition, by comparing the studies on castration and androgen replacement in young male mice, we also found that the effects of TP supplementation were significantly weaker in KO-GDX mice than WT-GDX mice, and the oxidative stress state of the SN was significantly lower in WT-GDX mice than KO-GDX mice. Thus, the present results that Nrf2 deficiency attenuated the efficiency of TP supplementation was to a certain extent due to high oxidative stress state. Activation of Nrf2 to reduce oxidative stress was beneficial for testosterone to play its ameliorative effects on mitochondrial dysfunction in aging and aged-related neurodegenerative disorders in males. Considering the involvement of Nrf2 in regulating mitochondrial functions, it was presumed that Nrf2 might also affect mitochondrial dynamics through direct or indirect way in TP supplementation to aged male mice. However, direct experimental evidence for Nrf2 roles in mitochondrial dynamics is limited [11]. So, it was unclear to what extent changes in mitochondrial dynamics in the present studies were involved in the improvement of mitochondrial function by TP. The changed mitochondrial dynamics in TP-treated aged male mice was causal in improved mitochondrial function, or merely a downstream consequence should be further clarified in the future study.

5. Conclusion

In summary, Nrf2 knockout further reduced the nigrostriatal dopaminergic neurochemical content and aggravated downregulation of both mitochondrial biogenesis and mitochondrial dynamics in the substantia nigra upon aging. Supplementation with testosterone induced the amelioration on nigrostriatal dopaminergic activity and mitochondrial function in aged male animals by modulation of mitochondrial biogenesis and mitochondrial dynamics. Nrf2 deficiency blocked testosterone-induced upregulation of mitochondrial biogenesis and mitochondrial dynamics in the aging substantia nigra. Thus, as a target, activation of Nrf2 might be conducive to testosterone-upregulating mitochondrial biogenesis and mitochondrial dynamics in the substantia nigra upon aging to produce mitochondria that are more efficient in ATP production and have optimal oxidative capacity.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

BR, TZ, QG, JC, and YK performed the experiments. BR and TZ wrote the manuscript draft. YW, RC, XJ, and GZ analyzed the data. GS designed the experiments and revised the manuscript. All authors approved the final version of the manuscript. Baoliang Ren and Tianyun Zhang contributed equally to this work.

Acknowledgments

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Supplementary Materials

Table S1: accession numbers of the genes for primers. *(Supplementary Materials)*

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Review Article

New Metabolic, Digestive, and Oxidative Stress-Related Manifestations Associated with Posttraumatic Stress Disorder

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Posttraumatic stress disorder (PTSD) represents a pressing and generally invalidating syndrome that is triggered by a terrifying or stressful experience, relying on recurrently reliving the traumatic event feelings associated to it, which is subsequently linked to ongoing activations of stress-related neurobiological pathways and is often associated with neurodegeneration. In this paper, we examine what lies beneath this disorder, reviewing evidence that connects PTSD with a wide array of mechanisms and its intertwined pathways that can lead to the decompensation of different pathologies, such as cardiovascular disease, gastrointestinal ailments, autoimmune disorders, and endocrine diseases. Also, the significance of the oxidative stress in this frame of reference is debated. Thus, knowing and identifying the main features of the distressing experience, the circumstances around it, as well as the neuropsychological and emotional characteristics of people prone to develop PTSD after going through disturbing incidents can offer an opportunity to anticipate the development of potential destructive consequences in several psychological dimensions: cognitive, affective, relational, behavioral, and somatic. We can also observe more closely the intricate connections of the disorder to other pathologies and their underlying mechanisms such as inflammation, oxidative stress, bacterial overgrowth syndrome, irritable bowel syndrome, metabolic disorders, oxytocin, and cortisol in order to understand it better and to optimize the course of treatment and its management. The complex foundation PTSD possesses is supported by the existing clinical, preclinical, and experimental data encompassed in the current review. Different biological systems and processes such as the hypothalamic-pituitary-adrenal axis, sympathetic nervous system, oxidative stress, inflammation, and microbiome suffer modifications and changes when it comes to PTSD; that is why targeted therapies exert tremendous alleviations of symptoms in patients diagnosed with this disorder. Therefore, this implies that PTSD is not restricted to the psychiatric domain and should be viewed as a systemic condition.

1. Introduction

In current times, we face a great need to study posttraumatic stress disorder (PTSD) together with targeted intervention strategies—a fact determined by events that transpire all around the world, such as population migration, an increasing number of separated families, and high divorce rates, but also unemployment, a poor socioeconomic status, financial issues, marital problems, abusive relationships, chronic stress, a violent climate in the household, psychological harm, fires, road accidents, and crimes against minors and their families.

Scenarios of extensive stress can inflict harm to both the adult and children, ultimately leading to PTSD, a syndrome commonly linked to different mental afflictions, such as major depressive disorder, anxiety and panic attacks, and several dependencies. Children, in particular, fragile as they are, are more prone to develop posttraumatic stress disorder,

being susceptible to a wide range of psychological modifications. Therefore, the severity of the disorder requires a complex approach to the problem [1].

Over time, stress has been perceived from three perspectives: (1) stress acting like a stimulus, or a critical event in life, which triggers the physical and mental reaction to stress, such as anxiety or cardiovascular problems [2]; (2) stress as a psychological and physical reaction to acute or chronic strains [3]; and (3) stress as an ongoing process between the person and his or her environment (e.g., addressing Lazarus's transactional stress) [4]. Of these approaches, the last one was the most appraised, as observed by Randall and Bodenmann [5].

In this way, Selye [6] described the general adaptation syndrome (G.A.S.), in which he suggested that the body reacts in a very similar way to a wide range of harmful stimuli. According to Selye [6], G.A.S. encompasses a vast array of response reactions of the body through which it defends itself against stressors (from a microbial infection to a strong emotion). G.A.S. comprises 3 phases: the "alarm reaction" stage, the "stage of resistance," and the "stage of exhaustion." The majority of processes happening during the AR stage, for example, a catabolic state, hypoglycemia, digestive wearing away, release of adrenaline, and hemoconcentration, revert to their prestress levels and functioning in the second stage. However, ongoing stress starts off the final stage, where the phenomena mentioned in the AR stage emerges back again [6]. During the alarm reaction stage, initially characterized by shock and decreased resistance to stressors (i.e., heightened senses and increased heart rate) is therefore designed to prepare the body to cope with stressors [3]. In the countershock phase, the biological defense reaction begins, manifested by increased respiratory rate, muscle tension, blood pressure, blood sugar levels, hormones, and cortisol in the bloodstream [3]. If the stimulus ceases, the body returns to its original balance [3, 6].

If its action continues, the body enters the stage of resistance, in which the body actively copes with the stressor. Defensive reactions intensify, and the acquired adapting process is maintained [3, 6]. If the stressor ceases, it returns to its original balance, but if it holds for too long, the body becomes depleted. The exhaustion stage is reached when the person has failed to adapt, when the subject can no longer defend himself. In this case, physical or mental illness sets in.

The stress defense mechanism is represented by processes that unite cognition and behavior and their physiology [7], acting together as a shield against the vicissitudes of the outside world, thus restoring homeostasis and ensuring survival of the species (Table 1).

The brain mediates the assessment and acknowledgment of environmental stressors but also other stimuli in order to establish and induce cardiac and vasculature adjustments, as well as adapt immunologic and endocrine processes [9]. Specific molecular alterations are made during these procedures, leading to changed neural pathways, efflux of chemical mediators, and the ultimate remodel of physiology [9]. Brain regions that play a part in the stress response encompass structures like the hippocampus, amygdala, and prefrontal cortex. The hypothalamus, which activates the sympathetic nervous system (SNS), leads to the release of adrenaline into the bloodstream, but also cortisol through the stimulation of ACTH hormone [10]. Also, the prefrontal cortex, the most evolved area of the brain, acts as a control center by handling emotional responses, thoughts, and actions. The brain stem, another key component, manages autonomic control but also how the body reacts to stressful stimuli. The amygdala, the so-called "brain's threat detector," belongs to the limbic system and is known for managing emotions, processing threats, modulating fear responses and behavior, and consolidating memories [11]. Lastly, the striatum, found in the center of the brain, controls cognition and reward and coordinates movements [12]. Sudden distress triggered by a chain of events as well as prolonged nervous tension after being subjected to multiple or recurrent traumatic experiences precipitates the progression of psychological warfare and deteriorates interpersonal skills, subsequently engaging psychopathology [13].

Regarding the frequency of this disorder across the world, research concerning epidemiology shows that roughly 3/4 of citizens will encounter a distressing experience, but solely 1/5 of the people who experienced major stress are prone to be diagnosed with posttraumatic stress disorder [14]. This syndrome has a wide range of manifestations, mostly parted in three categories: reexperiencing symptoms, avoidance symptoms, and hyperarousal features [15] (Figure 1).

The remarkable trait of the pathophysiology is that the fear response cannot be suppressed, and therefore, people with posttraumatic stress disorder relive distressing recollections, not being able to hold back the physiological reactions when dealing with traumatic impulses. Occurring modifications in neuroendocrine systems as well as modulation of metabolism, inflammation, or neurotransmission were illustrated in patients suffering from PTSD [17]. Furthermore, the stress disorder is linked to a range of different psychiatric conditions (for example, anxiety and panic disorders, depression disorder, impulse control disorders, addiction to various substances, legal/illegal drug abuse, and suicidal behavior) and somatic ailments and complaints (for example, heart and blood vessels, thyroid, pancreas, digestive, lung, muscular, and dermatological diseases) [18, 19].

2. Somatization Correlated with PTSD

Most of the time, those who have been through traumatic situations encounter different modifications related to physiology, onset or aggravation of somatic ailments, unexplained symptoms, somatization disorder, and other somatoform disorders [20, 21].

Somatic symptom disorder is depicted as an exaggerated concern over physical symptoms, such as weakness, pain, headaches, or tremor, which usually leads to emotional discomfort and troubles in daily life functioning [22].

Symptoms can vary from localized sensations, such as pain in the chest area or pain in joints, to generalized symptoms, such as dizziness, movement disorders, or weakness; they can also have no identifiable medical cause, or they

Table 1: Behavior and somatic adjustment in the stress process [8].

Adjustment of behavior	Adjustment of somatic processes
Modifying the behavior according to the situation at hand	Redirecting the energetic force of the systems involved according to their necessity
Altered sensory threshold	Directing the needed compounds (O_2, carbohydrates, lipids, proteins, vitamins, water, and minerals) towards CNS and other involved areas
Sharpened memory and sensation	Modified cardiac activity; elevation of blood pressure, heart rate, and force of cardiac contraction
A high sense of arousal and awareness	Elevated respiratory rate
Increased cognitive functions, attentiveness, and concentration	Increased gluconeogenesis+lipolysis
Suppression of behaviors related to nutrition (obtaining/consuming food)	Removing toxic compounds
Inhibition of reproductive functions	Freezing reproductive and growth axes
Inhibition of gastric motility; greater movement in the colon	Limiting the reaction to stress
Limiting the reaction to stress	Containment of inflammatory/immune response
Stress-induced analgesia	Release of norepinephrine and epinephrine

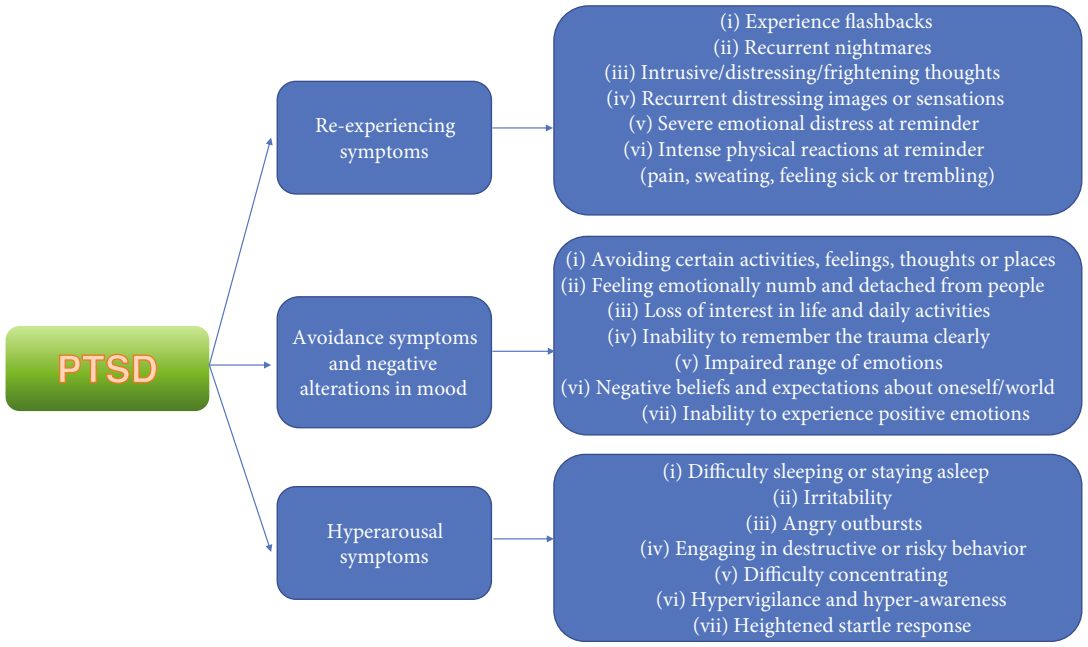

Figure 1: Symptoms of PTSD [16].

can be related to a specific medical issue but with a higher intensity than expected [22].

Somatoform disorders are represented by physical symptoms that do not possess a medical explanation and unlike dissociative disorders; their onset is progressive, as the patient is constantly addressing the medical services for further examinations and investigations, despite not finding a somatic cause or justification [23].

Complaints can involve any part of the body, but most often, patients present with digestive ailments (abdominal distress, bloating, nausea, vomiting, diarrhea, and constipation) and also dermatological symptoms, such as local/generalized pruritus, urticaria, burning, and numbness [23]. Also, the somatization disorder is more commonly found in women, with its onset happening in their 20s. More so, there have been reported multiple severe situations which frequently side with impairments of personal, relational, and professional life.

Patients who suffer from posttraumatic stress disorder often presented with somatic afflictions which cannot be explained by organic pathology, like blurry vision, unexplained vertigo, tinnitus, and somatoform disorders [24]. They can also display a variety of medical conditions such as cardiac, lung, neurological, muscular, digestive, immunity, and gynecological disorders; generalized physical complaints; chronic pain; diabetes; and sleep disturbances. This pathology encompasses limbic instability as well as modifications in the HPA axis and sympathoadrenal medullary

axis, which subsequently can alter neuroendocrine activity, as well as immunity, leading to autonomic nervous system dysregulation, pseudoneurological symptoms, and dysfunctions in sleep dynamics [24].

Even though a past filled with traumatic experiences is firmly essential to the existence of somatic symptoms, but not everyone who experiences distress in their lives is prone to psychological disturbances and somatization. A number of reasons for this variability involves diversity of genes, personality types, and demographical factors (for instance, more than 30% of soldiers diagnosed with posttraumatic stress disorder display serious physical symptoms, while the rest of them show no sign) [25]. Moreover, according to past observations, females displayed a predisposition towards developing somatic symptoms, such as nausea, tremors, pain, fainting, dizziness, headache, stomachache, and vomiting. Nevertheless, the intricate mechanism that connects trauma exposure to adverse mental health is only partially understood.

The link that connects terrifying experiences at a tender age with the development of somatic symptoms was revealed by researchers, including Heim and colleagues [26], who acknowledged a connection among abusive relations in early life and a physical ailment called "chronic fatigue syndrome." In a similar fashion, Waldinger, together with his associates [27], realized that children who experience persistent trauma become more vulnerable and develop somatic symptoms, while they also form insecure attachments over time.

Chronic PTSD, especially complex PTSD, was linked to a high rate of autoimmune conditions, including Hashimoto's thyroiditis, type 1 diabetes, and Crohn's disease. It was Boscarino [19] who postulated that "biological mediators of these conditions might have a clinical correspondence with higher T cell counts, higher IgM levels, hyperreactive immune responses on delayed cutaneous hypersensitivity tests, and also lower dehydroepiandrosterone levels."

Another frequent comorbidity among patients diagnosed with PTDS is cardiovascular disease [28]. A number of researchers illustrated that manifestations of posttraumatic stress disorder are connected to coronary heart problems. Chronic sympathetic arousal emerging from anxiety is crucial for the progression of cardiac disease employing reduced heart rate variability [28]. Other points of order lean towards high action of the SNS and low one from the PNS, universally known for causing the most perilous abnormal heart rhythms: ventricular fibrillation and ventricular tachycardia and ultimately cardiac arrest. Stress disorder presents a higher vulnerability for the development of cardiac disease, identifying a faulty pattern of AV transmission and also necrosis on the electrocardiography. On the other hand, depressive disorder is linked to surfacing arrhythmias [29].

3. The Interrelation between Gut Microbiome and Other PTSD-Related Distresses of the Gastrointestinal Tract

The individual microbiome has recently come under the spotlight for its potential contribution to individual variability in risk of developing posttraumatic stress disorder after having been exposed to stress due to its various interactions with the host, including effects on neural, neuroendocrine, and immune signaling [30]. The gut microbiome has been hypothesized to influence the cerebrum and cerebral activity in a matter of managing neurotransmission, generating compounds which regulate immunity functions brain and also toxic substances released by bacteria.

The intestinal microbiome consists of all microorganisms and their genes located in the gut, while "gut microbiota" refers solely to the living microorganisms (archaea, bacteria, viruses, and eukaryotes, such as fungi) [31]. The compositions of individual microbiomes emerge through an intricate interplay between genetics and environment, with the latter playing a dominant role into the story. The dynamism of the microbiome is defined by its ongoing change depending on lifestyle factors.

It is specified in the literature that at least one thousand organisms of the bacterial biomass colonize the human gut with at least 160 different species per individual, and cumulatively, these bacteria store an estimate of 150 times more genetic information than the human host [32].

Where digestion occurs, the bacterial phyla *Firmicutes* and *Bacteroidetes* encompass around 3/4 of the intestinal population, the two species being particularly sensitive to change [33]. Alterations of the microbiome are becoming more and more linked to the vulnerability towards allergies, disorders that target the immune system, diabetes, and psychiatric and neurological ailments, which have a widespread presence among people from all around the world.

As stated before, stress, emotions, and trauma have the power to modify the bacterial composition in the digestive tract. Cortisol and epinephrine interfere with the proliferation of bacteria, causing harm to the gut mucosa and therefore leading to an outflow of toxins and bacteria into the bloodstream. This process gives rise to inflammation, an etiology factor incriminated in various psychiatric disorders [33]. Research shows that gut bacterial biomass takes a toll on brain functions, thought processes, the ability to memorize, and also behavioral actions, sociability, and coping with emotional tension [31].

Knowledge is that the central nervous system controls gastrointestinal function in the gut, but it is partly clear how the gut habitat, including the microbiota, may influence brain function, especially in the realm of psychiatric disorders, such as anxiety and depression [31, 33]. Brain-gut-microbiome signaling pathways, including efferent neural, neuroendocrine, and immune pathways engaged by the central nervous system, work side by side to mediate homeostatic responses in the gut [30].

The possibility to develop PTSD as well as the symptom constancy may be influenced by the bidirectional signaling of the microbiome-gut-brain (MGB) axis. Alterations in microbiota proved to "modulate plasticity-related, serotonergic, and GABAergic signaling systems in the central nervous system" [32].

The gut-brain axis (GBA) refers to dialogues happening both ways amid the central nervous system and the digestive tract (the enteric nervous system), with the purpose of

monitoring and integrating intestinal activities and also engaging the cerebral areas responsible for cognition and emotions and linking them to the gut features, namely, intestinal permeability, "enteric reflex," immune activation, and enteroendocrine signaling [33].

This complex communication network, known as the GBA, comprises a vast number of mechanisms and elements such as the hypothalamic-pituitary-adrenal (HPA) axis, which is a fundamental component in how the body responds to stressful stimuli, immune cells, such as cytokines and chemokines, the vagus nerve, which is primarily involved in the mind-body connection, short-chain fatty acids (butyrate), neurotransmitters, and neuropeptides like serotonin (5-HT), GABA, dopamine, leptin, melatonin, histamine, and acetylcholine [34]. With tryptophan being the major serotonin precursor, interference with its metabolism represents a key factor, as more than 90% of serotonin is released by enterochromaffin cells found in the gastrointestinal mucosa. Serotonin plays an essential role in the brain, where it modulates mood, cognition, anxiety, and the learning process, as well as in the gut, impacting bowel function by modulating secretion, influencing motility and pain perception. Other components of the GBA are represented by gut permeability, which ensures the transportation, absorption, and balance of nutrients and also immunity, gut microbiome, and autonomic nervous system (ANS), engaging both afferent (sensory) signals, modulating gut motility and pain perception through calcium-dependent potassium channels and also efferent (motor) signals [33, 34] (Figure 2).

The GBA relies on reciprocity, as the central nervous system influences the processes occurring in the digestive system through motility, secretion, nutrient delivery, and microbial balance, while the gut also modulates how the brain operates, impacting mood, behavior, stress, and anxiety. Microbiota interacts with GBA through various mechanisms, primarily by regulating the intestinal barrier, which, if altered, influences and affects all the fundamental compartments.

Dr. Stefanie Malan-Muller conducted a study where she analyzed the gut microbiomes that belonged to individuals suffering from posttraumatic stress disorder. Afterwards, she gathered the same samples from people who have also had some tough life experiences, without developing the disorder (trauma-exposed controls) [31]. The team compared the two lots as they found a preponderance of 3 bacterial species, namely, *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia*, with significant differences in the PTSD lot (scarce number of the aforementioned species) in comparison to the other lot [31]. However, a reduced number of *Actinobacteria* and *Verrucomicrobia* were found in samples taken from people who faced adversities in their early years [31]. It is noteworthy to say that adults who faced childhood adversities are more likely to develop this disorder at some time in the future, having already set in motion a chain of events happening at the site of the microbiome in response to what they have previously experienced.

Furthermore, scientists have revealed how early-life stressors can alter the gut microbiome [35]. Emotional tension and HPA axis dysfunction can affect or induce a condition called SIBO (small intestinal bacterial overgrowth) through several mechanisms, such as a scarcer gastric acid secretion, a slower digestive motility, altered level of secretory immunoglobulins, a bigger vulnerability towards pathogens, an increased virulence, and the accumulation of biofilm [36].

SIBO is often deemed responsible for poor absorption of food nutrients, as well as chronic diarrhea, being characterized by the invasion of colonic bacteria proximally into the ileum and jejunum, with the bacterial population exceeding 10 [5]–10^6 organisms/mL (the standard multiplied by a thousand) [37–40]. By competing for fundamental nourishment, the bacterial flora is likely to alter the host's metabolism, impacting the mucosal lining of the individual, as well as generating digestive phenomena that lower/change dietary consumption [38].

Poorly absorbed nutrients (malabsorption) are a typical feature of this condition, with the possibility to lead to different ailments in the individual. Individuals who suffer from bacterial overgrowth could equally experience unexplained slimming, as well as vitamin deficiency (B_{12}—leading to anemia, vitamin D—causing osteoporosis, and vitamin A—vision impairment). This condition is named blind loop syndrome [37].

SIBO generally occurs due to faulty or abnormal behavior in systems which are trying to maintain homeostasis by controlling gut microorganism colonies. Reduced secretion of gastric acid and dysmotility in the small intestine are often associated with predisposition to bacteria overcrowding [37], as well as abnormalities in the anatomy of the GI tract and immunity impairment, which can amplify the risk for bacterial overgrowth. In time, their accumulation can result in inflammation of the gut mucous membrane, which ultimately aggravates the typical symptoms of SIBO [37].

The prevalent symptoms include pain or discomfort in the abdomen, diarrhea, fatigue, meteorism, weakness, and excessive gas [41]. Several patients only mention one or two complaints; they can either present with vitamin deficits or just a few dropped pounds. Others experience explosive diarrhea or fatty stools. Symptom frequency and severity directly correlate to the level of overpopulation, as well as the gastric mucosal degree of impairment [37].

The mechanism through which the risk of developing SIBO increases holds accountable an impaired migrating motor complex (MMC) [36]. By prompting food to linger in the small intestine, it provides a favorable and nurturing environment for bacterial populations to increase abnormally. The MMC is a periodic coordinated movement of myoelectrical waves, which uses contractions of the GI smooth muscles during fasting and has a glaring effect on the peristalsis through the GI tract [42]. It is known to play a "decluttering" part by moving undigested residue through and out of the GI tract [42]. Evidence suggests that stressful situations act as direct inhibitors for MMC [43]. Beaumont postulated that anger, fear, or any emotions whatsoever that devitalize or disrupt the nervous system were linked to suppression of digestive motility and altered digestive function [44]. It is known that corticotropin-releasing factor (CRF), a fundamental regulator of the HPA axis, mediates

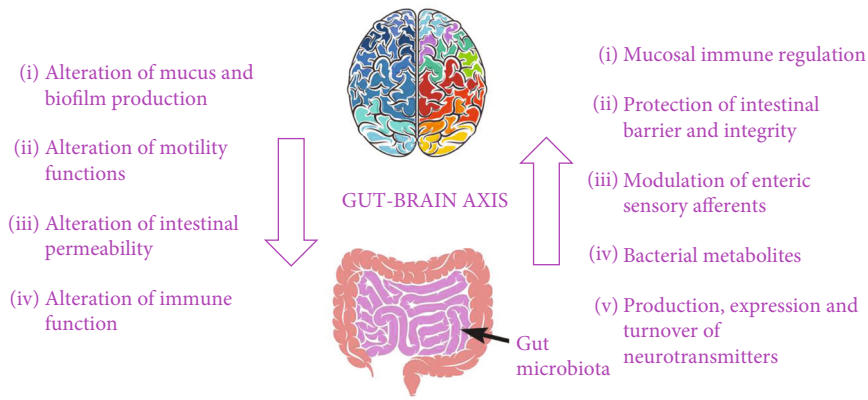

Figure 2: The gut-brain axis and principal mechanisms of bidirectional communication that happens amid the central and the enteric nervous system [33, 34].

the suppression of the MMC (in times of nervous tension) [36]. Upon its release by the hypothalamus, corticotropin-releasing factor will bind to brain receptors, impairing the synaptic transmission governing the migrating motor complex [45].

Stress is yet another cause for gastrointestinal motility dysfunction, as it generates significant fluctuations in blood sugar levels [36]. Elevated levels of cortisol on account of prolonged stress produce glycemic fluctuations. These spikes and drops therefore encourage prolonged periods of hunger, resulting in more frequent meals (ergo increased food intake), ultimately reducing the wait time from one meal to the next, which is when the migrating motor complex is the busiest, therefore negatively impacting GI kinetics [36].

Moreover, stress response mediators, such as cortisol and catecholamines, set the ground for the aforementioned biofilm formation (represented by a population of microorganisms that share common DNA and nutrients, adapting skills such as eluding the immune system, acting like a shield against antimicrobial treatments, resulting in infections that are hard to cure), by aiding harmful bacteria retrieve nourishment as a means to survive [46–48].

Regarding the gastrointestinal distresses, another pathology comes to light when it comes to its connection with PTSD. IBS recognizes the existence of predisposing or aggravating factors such as psychosocial stressors in its onset [49]. Unlike patients with structural gastrointestinal disorders, FGID patients are more likely to have suffered emotional disturbance or to have experienced life-threating situations [49].

IBS or irritable bowel syndrome represents a functional bowel disorder, with its main features being recurring abdominal pain happening weekly for a three-month period and that is associated with two or more of the following: differences in the defecation process, a modification in stool appearance or a perceived difference in bowel habits (frequency) [50]. Around 1/5 of adult population as well as teenagers universally experience these symptoms and more, predominantly in women, as indicated by research [51, 52]. Patients usually experience an array of comorbidities such as meteorism, cramping, constipation/diarrhea or both, and bowel incontinence, but also emotional and social distress [50]. Irritable bowel syndrome has been linked to conditions such as anxiety, depression, and a stressful living situation, while considering the GBA indispensable for understanding IBS [53].

The intricate matrix of etiologies likely constitutes a variety of circumstances targeting pathophysiology, which may vary from one individual to another, including "visceral hyperalgesia," "leaky gut (intestinal permeability)," activation of the immune system, GI motility modifications, "autoimmunity," and alterations of intestinal microbiota [54].

As we previously stated, patients suffering from IBS are likely to be more vulnerable and susceptible to emotional and psychological turmoil, presenting a higher rate of comorbidities among mental disorders, including anxiousness and stress, but also low dynamism/vitality, poor quality of sleeping patterns, and performance difficulties on a daily basis [55]. That is why many treatments target neurobehavioral intervention and tackling the use of antidepressants.

A number of hypotheses postulated a potential connection between irritable bowel syndrome and PTSD. IBS has a multifarious pathogenesis, having psychological, social, hereditary, endocrine, central and enteric nervous system, visceral sensitivity, and hyperalgesia as well as infectious and/or inflammatory elements [56]. Tanaka et al.'s "biopsychosocial model" for the aforementioned disorder promotes this conceptualization [57]. Therefore, the circumstances influencing the onset and progression of IBS point to genetic factors and "social learning" as belonging to childhood and formative years, psychological and social events (mistreatment, emotional upheaval, stress, and mental frame of mind), and pathophysiological factors, such as alterations in terms of sensitivity and motility—stronger and longer contractions lead to meteorism and diarrhea—and also dysregulation of the HPA axis. Another key component is the communication between the brain and the digestive system, where defectively coordinated signals can trigger pain or bowel movement modifications. Even more, irritable bowel syndrome has the possibility to develop after a bacterial or viral infection of the digestive tract [54].

For instance, a study conducted by Ringel and his team [58] utilized PET scans in order to observe cerebrovascular

perfusion in people suffering from IBS in comparison to the control group, along with subsequent examinations and interpretations, differentiating individuals who presented a background of sexual assault or physical mistreatment as opposed to people showing no signs of abuse. The results showed a higher activity spike in the "cortex cingularis anterior" for individuals without irritable bowel disorder and for the people without a background of abuse. IBS patients were linked to greater thalamic activity, this region being responsible for nociception response.

Going back to the topic at hand, concerning dysbiosis, a number of studies have shown promising results. For example, in a mouse model displaying distressing pressure in early stages, mother estrangement results in microbial dysbacteriosis, enhanced HPA axis activation, and increased anxious reactions and protective/aggressive behavior later on [35]. Another study showed how maternal stress during pregnancy leads to increased concentrations of glucocorticoids (cortisol) determined in the saliva. This proved to actively impact the infant's microflora configuration and diversity, as maternal stress consequently altered the infant's microbiome throughout their first 4 months of life [59].

It is well established that people with mental health problems caused by stress, like anxiety and depressive disorder, exhibit altered microbiota abundance and higher gut permeability [60]. Animal models who experienced social defeat (ongoing stress) presented with a remarkable decrease in terms of relative abundance and general diversity of many bacterial species (such as *Akkermansia* spp.), as well as less frequent signal transduction pathways, such as seven-transmembrane domain receptors that undoubtedly correspond to quantitative assessment criteria found in major depressive disorder [60]. Moreover, traumatic physical stressors are linked to major alterations in the gut flora, where the changes take place within 72 hours of the injury, leading ultimately to dysbiosis, higher gut permeability, and an elevated concentration of circulating proinflammatory cytokines [32]. Furthermore, activation of the HPA axis will lead to increased secretion of glucocorticoids, ultimately causing dysbiosis as well.

Another sine qua non element is the sympathetic nervous system, which, if stimulated for an extended period of time, can change microbial composition in the gut and increase gut permeability, caused by the increase of catecholamines released by the adrenal gland and sympathetic nerve terminals [61]. It was also demonstrated that epinephrine and norepinephrine could induce the proliferation of a variety of gram-negative bacteria, especially *Escherichia coli* [62]. These bacteria also proliferate after injury-induced release of norepinephrine, where the SNS is involved as fundamental player in intestinal colonization of gram-negative bacteria and gut dysbiosis [63].

As presented before, emotional upheaval is a fundamental trigger that influences the alteration of digestive microflora, as well as the intestinal mucosa barrier function. Changes to the intestinal microbiome on account of stress during childhood, a susceptible time when the digestive microflora configures the homeostatic immunity functions and the neurological pathways of the host, may carry deep-rooted repercussions regarding immunity, spiking the likelihood for subsequent illnesses related to stress in the future and contributing to a proinflammatory state and low cortisol in adulthood [32].

4. The Key Part Inflammation and Oxidative Stress Play in PTSD

Recent evidence has come forth suggesting that some PTSD outcomes combine high systemic degrees of oxidative stress (OXS) with inflammatory activation. Inadequate regulation of the immune system and increased inflammatory levels were found to be potential risk factors, while bacterial resources play a major part in immunoregulation [64]. On the other hand, complex posttraumatic stress disorder is a type of recurrent and long-term trauma which increases oxidative stress and puts cell senescence into overdrive [65]. Where inflammation (INF) occurs, different biological catalysts are released, including reactive species of oxygen and nitrogen, proinflammatory cytokines, and other molecules, thus inducing oxidative stress. Oxidation and inflammation therefore have the tendency to happen at the same time, as both of these processes are likely to cause the onset of the other.

Inflammation is how the immune system responds from a physiological point of view to a variety of factors and injurious situations, including infectious agents, cellular injury, harmful substances, or irradiation [66]; the immune system reacts by fighting against pathogens, followed by elimination of cellular debris and ultimately launching of the recovering process [67]. INF is, ultimately, a crucial biological warrior and the key to survival and adaptation [68].

Triggers can produce acute or chronic inflammation in cardiovascular, hepatic, renal, pancreatic, respiratory, cerebral, digestive, and genital systems, having the potential to trigger tissular injuries and disease [69]. Inflammatory cells become activated by (non-)infectious agents, as well as cellular injury which eventually sets off inflammatory signaling pathways. As a reaction to tissue injury, the body fights back by initiating a chemical signaling cascade which will therefore stimulate responses in order to heal the damaged biological material. The signals trigger and attract leukocytes through chemotaxis, directing them from the bloodstream to the injured sites. After their activation process, leukocytes secrete cytokines, which regulate inflammation and also act as immunomodulating agents [70].

The action of different biological catalysts such as reactive species of oxygen and nitrogen, proinflammatory cytokines, and other mediating compounds leads to oxidation. Therefore, inflammation and oxidation as processes have the tendency to happen simultaneously, also being capable of inducing one another [65]. Altogether, these concepts can be triggered and severely impacted by psychological stress, especially when it occurs at length.

Regarding the neuroinflammatory component in the pathophysiology of PTSD, evidence suggests that considerably high levels of mRNA as well as specific mechanisms that produce cytokines were found in the brain [71]. Even more, the potency of oxidative stress extends past the brain,

involving the adrenal glands, as well as blood, proving that PTSD has the potential to develop into a systemic condition which involves multiple organ systems. Up-to-date studies have shed light on amplified inflammation processes found in the pathophysiology of posttraumatic stress disorder; for example, it was found that T regulatory cells (Treg) were modified in stress-afflicted patients. Treg play a key part in defending the body against inadequate inflammation effects, such as those encountered in autoimmune disorders, allergies, and asthma [72].

Furthermore, decreased amounts of Treg were found following the human participants' in-lab stressor subjection. To this point, reduced T regulatory cell frequency is linked to autoimmunity phenomena found in disorders like Hashimoto's disease, IBD, and rheumatoid arthritis, as people diagnosed with posttraumatic stress disorder presented an elevated risk for such conditions [72, 73].

In line with these discoveries, genome-wide association studies (which identify which genes are responsible for certain pathologies) on groups of people who suffered from posttraumatic stress disorder discovered an important connection with the Ankyrin Repeat Domain-55 gene, which is linked to a series of inflammatory and autoimmune diseases, such as MS, T2D mellitus, gluten-sensitive enteropathy, and rheumatoid arthritis [74].

Furthermore, posttraumatic stress disorder was found to cause upregulation of serum interleukin 6 (IL-6) and proinflammatory cytokines, such as interferon-gamma (IFN-γ), interleukin 1 β (IL-1β), interleukin 10 (IL 10), and tumor necrosis factor α (TNF α) [75–77]. Studies have shown that proinflammatory cytokines increase in concentration post exposure to stress, but also when dealt with secondary symptoms of stress, such as fatigue and sleep disturbances. High concentrations of CRP (C-reactive protein), an inflammation marker used clinically, were also found in individuals with PTSD, correlating with symptom severity [75, 78].

Glucocorticoid-mediated immunosuppression can lead to the short-term decrease of inflammation, but eventually may also trigger a homeostatic inequity among the mucosa, digestive microflora, and pathobionts (potentially pathological microorganisms). Cortisol does cause a boost in commensal pathogens like Helicobacter pylori, bacteria known to amplify local and systemic inflammation and lead to chronic ailments like gastritis [79, 80].

In an experiment, animal models subjected to negative psychological stimuli display an increase in Helicobacter species, assessed via RT-PCR to determine absolute/relative abundance; in order to prevent this effect, it was recommended to administer an antiglucocorticoid [81, 82]. Helicobacter spp. proved to cause inflammation in the inner lining of the colon in IL-10−/− rodents, with inadequate regulation of the immune system, a potential effect of the exaggerated immune defenses of the host [83]. The inoculation process using a tyndallized preparation based on bacteria with immune-regulating properties that boosts Treg and anti-inflammatory cytokine levels proved to avert stress-induced spikes in a disease similar to posttraumatic stress disorder in rodents, which would suggest that tipping the scales of pro- and anti-inflammatory elements might play a significant role in developing a PTSD-like syndrome [81–83].

Oxidative stress is a phenomenon attributable to a disturbance in homeostatic balance between pro- and antioxidants resulting in the production of reactive oxygen species (ROS), which have predominantly beneficial effects to cells, except for the time when they exceed their basal level. Certain mechanisms that promote oxidation will trigger molecular signaling pathways, consequently generating free radicals, as well as reactive nitrogen species (RNS) [84].

OXS happens when levels of ROS (superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl, nitric oxide, and reactive aldehyde or other prooxidant molecules) are so high that the present antioxidant molecules (superoxide dismutase, glutathione) cannot counterbalance the repercussions. Reactive oxygen species cause harm by oxidizing proteins/enzymes, carbohydrates, and lipids, as well as DNA or RNA and other cell elements, leading to functional cellular alterations, tissue necrosis, and upregulation of proinflammatory cytokines. Oxidized proteins will present themselves with the prospect of substantial cytotoxic consequences. When it comes to the homeostasis of cells, there are certain physiological fluctuations regarding the cellular redox state; however, an increased level of oxidative stress will lead to pathological modifications of cell signaling, thus gifting the cell with an inflammatory phenotype. OXS is an elementary biochemical senescence process, as the capacity of the body to counterbalance it is crucial for ensuring somatic welfare, lastingness, vitality, and endurance [85]. Furthermore, oxidation sets off signaling pathways that promote inflammation (and the other way around) [86, 87] and is involved in the etiopathogenesis of several health problems, like cardiovascular disease, chronic obstructive pulmonary disease, cancer, diabetes, and neuropsychiatric or neurodegenerative conditions such as psychotic disorder, Alzheimer disease, amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis, autistic spectrum disorder, and bipolar disorder [88–91] (Figure 3).

Inflammation and oxidative stress represent key parts inside the pathogenesis of neurodegenerative disorders, as well as psychiatric ones. Research shows that phytochemicals such as polyphenols (flavones, flavonols, flavanones, flavanols, stilbenoids, anthocyanins, etc.) that are usually found in compounds such as cocoa, olive oil, grapes, berries, coffee, tea, and peanuts could exert a positive effect on the intrinsic mechanisms of these disorders on account of their antioxidative effects [91].

Recent evidence shows that psychological disorders that develop after exposure to stress, such as posttraumatic stress disorder, as well as persistent emotional tension, can trigger and influence the concept of oxidation and inflammation, which means that their chronic effects and exposure have destructive consequences on the brain [92].

Of all structures, the cerebrum harbors the biggest vulnerability and susceptibility to the effects of oxidative stress due to its elevated metabolic demand, increased use of carbohydrates and O_2, and dense composition of oxidation-prone lipids [93]. In the central nervous system,

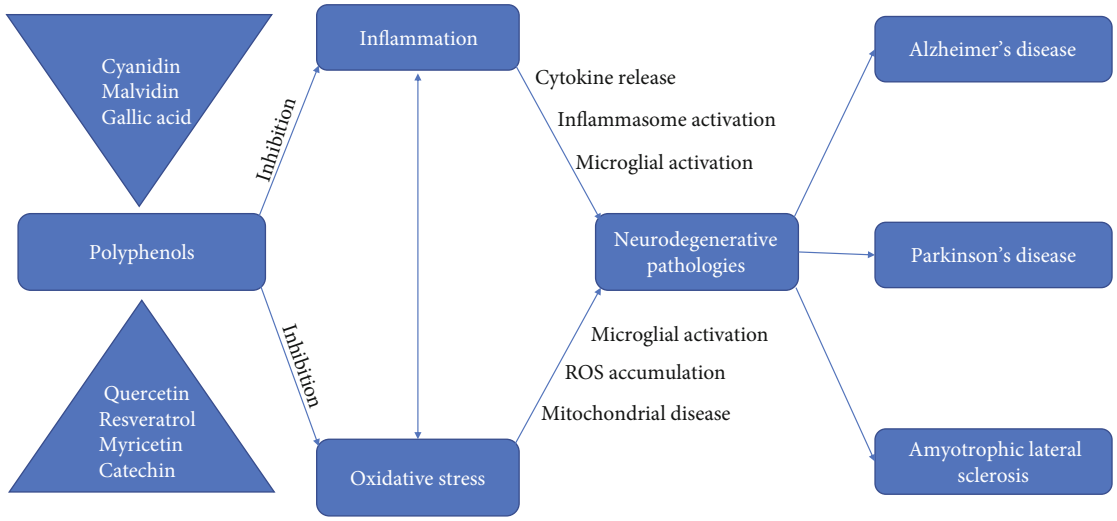

Figure 3: Polyphenol inhibition of neurodegenerative mechanisms [91].

the consequences of OXS are represented by a higher blood-brain barrier permeability, difficulties in neurotransmission, defective synaptic plasticity, disruption of neurogenesis, altered patterns of neural growth, and remodeling of neural morphology. It is also well established that OXS takes a toll on neural cell destruction and lesions in neurological conditions such as Parkinson's and Alzheimer's disease [94].

High levels of oxidation stress serum biomarkers were also found in healthcare workers who have experience prolonged, intensive nervous tension, with significant degrees of apprehended tension linked to increased extent of nucleic acid and lipid oxidative injuries [95]. Research proved that in overburdened people, the connection among the stress they felt and the injuries oxidative stress inflicted was mediated by cortisol [95]. Similarly, inquiries conducted on individuals manifesting anxious behavior revealed increased degrees of lipid peroxidation in subjects with generalized anxiety disorder [96] and inhibited antioxidative activity in subjects with panic disorder [93]. There have also been studies that suggested that individuals diagnosed with depression display inhibited antioxidative activity and high extent of nucleic acid oxidative injuries [97].

Several animal models involving rats postulated that a prolonged exposure to stress leads to increased ROS levels in certain regions of the brain, namely, the prefrontal cortex and the hippocampus. Scientists also observed features such as weight gain, a higher body temperature, and changes in the liver and heart histopathology depicting tissues with inflammation and fibrosis [98]. They also found a significant level of OXS-related proteins in plasma, as well as a high inflammation state.

When it comes to ailments of the gastrointestinal tract, the mucosa acts as a protective barrier, which can be overpowered by microbial pathogens and ingested compounds, triggering inflammatory responses, but also oxidative injury at the site. Disorders such as peptic ulcers, neoplastic diseases of the gastrointestinal tract, or inflammatory bowel disease have pointed to oxidative stress as an underlying mechanism of the pathogenesis [99].

As previously discussed, ROS are produced within the gastrointestinal tract and they serve as indispensable signaling molecules. For example, they are mainly used by cancer therapy, such as chemotherapy or radiotherapy, in order to induce apoptosis and ultimately remove malignant cells. However, the body homeostasis can be easily disturbed when there exists a disproportion of ROS generation, as they are largely fabricated with regard to agents such as cigar smoke, alcoholic beverage intake, administration of NSAIDs, or processes such as infections or ischemia-reperfusion (I/R) injury [100].

Inside the GI tract, ROS are predominantly generated by two enzymatic reactions: the hypoxanthine/xanthine oxidase system and the NADPH oxidase system. Xanthine oxidase turns hypoxanthine in xanthine and, later on, to uric acid (both reactions generating O_2^-) [100]. In the ischemia process, there is an increased production of xanthine and xanthine oxidase, at the expense of antioxidant enzymes, therefore generating ROS species such as O_2^- and H_2O_2, leading to an impaired digestive system [100]. A disruption in the GI tract barrier will lead to an altered gut permeability, ultimately contributing to inflammation by stimulating PMNs.

Moreover, emerging studies suggest that neurological degeneration related to posttraumatic stress disorder might have something to do with the condition's extent and intensity, meaning that the neuronal repercussions are directly proportional to the period during which a patient experiences the aforementioned disorder [93]. Phenomena such as flashbacks and intrusions are associated with activation of neuronal circuits related to fright, an amplified catecholamine and cortisol output, and also an increased peripheral autonomic nervous system activity.

Research based on structural brain imaging discovered clear correlations amid posttraumatic stress disorder and shortfalls of neuronal wholeness in places such as the amygdala, hippocampal region, and the medial prefrontal cortex, as well as the anterior cingulate cortex [94].

The hypothalamic-pituitary-adrenal axis is a fundamental physiological pathway, ruling the stress response system.

Alterations of its neuroendocrine functions proved to be associated with the pathophysiology of PTSD [101], and its long-term incessant stimulation impacted negatively the cerebral structures.

"The glucocorticoid-hippocampal atrophy model" [102] states that stress-related secretion of glucocorticoids (GC) leads to a degree of neurotoxicity at the CNS level. Because of its abundance regarding GC receptors, the hippocampus is explicitly at ease. A variety of research performed on animals revealed that high concentrations of GC are linked to an elevated number of reactive oxygen species as well as injuries resulted from oxidation.

Another common symptom of PTSD is represented by sleep disturbance, on which OXS has a resounding effect if we take a look at what lies beneath. The sleeping process is fundamentally important for the cerebral structures and mechanisms when it comes to detoxification and restoration. This can manifest as recurring night terrors, disruptive sleeping patterns, and dyssomnia or parasomnia [103, 104].

While sleeping, neuronal labor is diminished, thus favoring antioxidative processes [105]. This represents an essential process considering how sleep, through its five stages, restores the mind and body, as revealed by drops in antioxidant agents and remarkable spikes in oxidative biological markers following lab-induced sleep deprivation [106, 107]. Similarly, sleep deprivation has led to higher proinflammatory molecule concentrations (TNFα, IL-6), as well as C-reactive protein. As it is known, the sleeping process is crucial for sustaining memory and cognitive functions, as well as learning, cerebral detoxification, and encouraging neuronal rehabilitation [105, 108]. Extended wakefulness periods lead to a cerebral abundance of reactive oxygen species because of increased conversion of oxygen into energy [109]. There have also been animal studies that support the hypothesis according to which regular poor sleep triggers oxidative stress in the hippocampal area, thus leading to shortcomings when it comes to memorizing. Therefore, by using antioxidants, this effect can be counterbalanced [110].

To sum up, evidence from across a variety of studies (clinical, neuroimagistics, etc.) supported the premise that complex posttraumatic stress disorder is correlated with increased levels of oxidative stress [65]. Moreover, oxidative stress together with inflammation develops progressively alongside the aforementioned disorder, thus providing a modern perspective over therapeutic interventions.

5. Metabolic Disorders and Their Connection to PTSD

Clinical data backs up the hypothesis according to which beneath the impairment of fright suppression functions, posttraumatic stress disorder also represents a metabolic disorder on the basis of modified function of inflammatory response mechanism, involving neurological pathways like the hypothalamic-pituitary-adrenal axis, SNS, and inflammation [17].

The metabolic syndrome (syndrome X) is characterized by the succeeding clinical features: excessive body fat around the waist (central obesity), high fasting blood glucose (>100 mg/dL), hypertension (>130/85 mmHg), high triglyceride concentrations (>150 mg/dL), and low HDL cholesterol concentration (<40 mg/dL) [111]. Syndrome X as well as an increased body mass possesses a low responsiveness to leptin (a hormone released from the adipose cells), which leads to elevated plasma leptin concentrations and leptin insensitivity [112]. Before receiving its new name, this disorder was once called "insulin resistance syndrome" on the account of how important was this characteristic. An elevated blood sugar level happens in parallel with insulin resistance, as cells fail to react in a proper manner to insulin, thus leading to hyperglycemia [113]. Research has shown that the posttraumatic stress disorder can tangle with obesity and accompanying metabolic dysfunction [114].

The frequent comorbidity that occurs among syndrome X, type 2 diabetes, obesity, and posttraumatic stress disorder implies there are intrinsic neurological and endocrine alterations, as well as changes in the metabolism regarding the psychiatric disorder which can heighten the likeliness of a "systemic metabolic dysregulation." It can also reveal a fundamental metabolic shift due to a stressful history [17]. Processes such as oxidative stress, heightened autonomic activity, glucocorticoid activation, or immunological dysregulation have been incriminated to lie at the foundation of this connection between PTSD and metabolic syndrome. Moreover, changes regarding pathways in inflammation subsequent to modifications in GC receptor responsiveness (secondary to emotional and physiological arousal) could represent the underlying basis for inappropriate social conduct concordant with posttraumatic stress disorder and the pathophysiological display of syndrome X [17].

A number of changes of the hypothalamic-pituitary-adrenal axis found patients suffering from posttraumatic stress disorder revert to "HPA axis-centric" modifications seen in dysmetabolic disorder, namely, excess body fat in the abdomen area [115]. As established before, the increased girth typical of syndrome X is linked to pathway modifications of the hypothalamic-pituitary-adrenal axis. As cortisol suppression is lowered [116], its reaction towards distressing stimuli is increased [117], as well as increased glucocorticoid levels observed at 7 a.m. [118, 119]. Thus, the heightened glucocorticoid activity and concentration are linked to hyperglycemia, insulin resistance, and high TG levels, key aspects for metabolic syndrome [120]. This being said, it suggests that the metabolic consequences resulting from the central stress response system joined by persistent emotional tension work synergically as a way of amplifying insulin resistance together with hyperglycemia formerly existing in metabolic syndromes [113].

The perceived modifications of the hypothalamic-pituitary-adrenal axis within the posttraumatic stress disorder are concurring with the activation of the SNS, therefore raising blood pressure and heart rate after subsequent exposure to stimuli of an acute stressor, thus acting as a predictor for the upcoming development of the illness [121].

Metabolic disorders are equally correlated with a heightened SNS activity [122, 123], such as enhanced muscle sympathetic nerve activity. Behavioral factors linked to PTSD such as unhealthy diet and insufficient exercise are likely to

contribute directly to metabolic syndrome. There is a possibility that psychological symptoms and cardiometabolic processes exert epigenetic modifications expressed in the brain. Signs and symptoms of metabolic disorders and PTSD might impact the sturdiness of the interface that mediates the interaction between blood and cortex by increasing its permeability, thus enabling the dissemination of inflammatory compounds. Processes such as INF within the nervous system as well as OXS will eventually lead to decline in cognition and neural functioning [124].

Additionally, a high leptin level was discovered in the biological profile of people who experienced trauma, as well as in individuals who suffer from obesity [125]. Another common aspect between metabolic disorder and PTSD is insulin resistance, as the latter is correlated with a slight elevation of insulin concentration, as well as higher insulin responsiveness after performing a glucose tolerance test [126]. Similarly, data shows that the peripheral metabolism in PTSD is disrupted as well as the central glucose metabolism [17].

Overall, as certain baseline features seen in the metabolic disorder can be noticed in the pathophysiology of posttraumatic stress disorder, this further highlights the interconnection amid the aforementioned pathologies. Viewing the duality of posttraumatic stress disorder, further medical care can be directed towards the common links, in order to help individuals reach physical wellbeing.

6. Hematopoiesis Disbalance and PTSD-Related Manifestations

As stated before, psychiatric disorders in many instances present themselves with an imbalance regarding the bacterial population within the digestive system. A state of dysbiosis has been linked to obesity, irritable bowel syndrome, and even autoimmune diseases. Lately, evidence has shown that the gut microbial population influences altogether the process of generating blood cells inside the human body. Therefore, hematological alterations and gut dysbacteriosis have been associated with different health problems, namely, inflammatory bowel disease or metabolic disorders [127]. Furthermore, dysfunctions involving the production of erythrocytes, lymphocytes, and myelocytes have recently been shown in the posttraumatic stress disorder, while also linking them to the inflammation process.

The formation of blood cells is influenced by both external components, namely, cytokines and proteins that promote cell growth, as well as internal factors, such as genetic and transcription service, coordinating the differentiation of stem cells [128].

In a study conducted on mice for a period longer than two weeks, scientists observed that a therapeutic intervention using broad-spectrum antibiotics depleted and altered the gut microbial flora, influencing altogether the number of stem cells and their descendants found in the medullary cavities of the bones. Ultimately, this leads to anemia, panlymphopenia, and leukopenia due to the reduction and modification of gut microbiota caused by antibiotic treatment [100]. However, these effects can be easily reversed using the technique of fecal microbiota transplantation.

Recent studies showed the critical role of lipopolysaccharides (microbial components) that uphold the production of neutrophils and expression of Toll-like receptor/MyD88-mediated signaling [99].

The commensal gut microbiota supervises the adequate immune performance and hematopoiesis by involving microbial elements such as lipopolysaccharides to assist a constant production of neutrophils that fight pathogens using Toll-like receptor/MyD88-mediated signaling [99].

Even more, studies revealed that alterations of the microbiota are responsible for how well the body responds to different oncological interventions, such as chemotherapy and immunotherapy [129].

In another study conducted by Josefsdottir et al., the team postulated that commensal gut microbes are involved in regulating and sustaining normal hematopoiesis [100]. The gut microbiota manages the migration and phenotype while also influencing how numerous innate and adaptive cells behave.

Microbes residing in the gut prompt macrophages, dendritic cells, and also lymphocytes from the mucosa, which, therefore, trigger multiple extrinsic stimuli. These cellular and microbial stimuli sustain the tonic activity of hematopoietic progenitor and stem cells, together with white blood cells, such as neutrophils [99]. This will ultimately boost hematogenesis and provide the necessary tools for the immune system, with both its specific and nonspecific branches, to fight pathogens and limit the spread of the disease.

Alteration of bacterial communities in the gut sets the ground for increased susceptibility to a series of disorders [130]. Treatments, such as systemic antibiotics, can modify the diversity and shift the bacterial balance within the microbiome, leading to a compromised hematopoiesis and a higher propensity for infections.

In addition to influencing the body's defense mechanism, the microflora is required in order to keep systemic communities of neutrophils in the blood circulation and CD4+ T cells in the spleen, implying that the microbial population residing in the digestive tract plays a part in the ontogenesis of the immunity biosecurity [131].

7. Oxytocin and Cortisol and Their Influence in PTSD

Oxytocin, a peptide hormone and a neurotransmitter, may likely be an instrument that links childhood emotional upheaval with dissociative disorders, somatic symptom disorders, and PTSD [132]. Adverse events from a tender age, such as maternal deprivation, might leave their mark on how well those people react to stress with its neurobiological process, correlating persistent high amounts of plasmatic glucocorticoids and an enhancement of their sensitivity and receptivity to a various array of stressors, thus involving cortisol in this pathology [133, 134].

Oxytocin is a hypothalamic nonapeptide, secreted by the pituitary gland, which, as both a neurotransmitter and paracrine hormone, plays a vital role while engaging

in a variety of biological processes such as the adjustment of digestive system functionality, female pregnancy, signal transduction, synaptic transmission, heart rate and blood pressure modulation, sleep, memory, and cognitive function. Even more, it possesses psychological effects by shaping emotion, social and romantic bonding, empathy, trust, and attachments.

Oxytocin also regulates reproductive functions, the birth process by stimulating the uterine muscles, lactation by inducing the contraction of myoepithelial cells in the milk ducts, and maternal and paternal behavior [132]. The oxytocin receptor is expressed in the hypothalamus and brainstem and in brain areas that process olfaction such as olfactory nuclei and piriform cortex, amygdala, nucleus accumbens, anterior pituitary, insula, and striatum [135].

On the other hand, cortisol represents a steroid hormone produced by the adrenal glands [136], thus being the final compound that results from the hypothalamic-pituitary-adrenal circuit. At the time stressor factors are taking over a person, his nervous system prepares an adequate activation response [137]. Concurrently, the HPA axis secretes high quantities of cortisol in order to adjust the body's functions. After the threat has passed, the axis proceeds by resetting its activity and reverting to its normal tone [138]. Nevertheless, if the stress exceeds and the body is stuck in a prolonged perceived threat situation, where large amounts of hormones are secreted, in the end, it will lead to a less reactive hypothalamic-pituitary-adrenal axis [139]. Furthermore, if the axis fails to revert to its standard function, unusual cortisol amounts can be found in individuals suffering from posttraumatic stress disorder [140]. As a consequence, we ask ourselves if it is possible to make a biological marker out of cortisol when it comes to people diagnosed with this disorder.

In general, soon afterwards a distressing experience (so-called "phasic" reactions), the production of cortisol is usually increased, a fact noticed in warriors who were attacked [141] and in women who suffered from rape [142]. On the contrary, baseline 24 h cortisol elimination was at a low point in individuals diagnosed with stress disorder in comparison with controls [143]. This can result from a higher epinephrine and cortical receptivity, acknowledging that in PTSD exists an enhanced dexamethasone suppressibility [144] or a lowered responsiveness of the adrenal cortex [145]. Another research study found a higher level of stress hormone in collected urine from a twenty-four-hour period in groups focusing on battle warriors suffering from PTSD [146], in females with stress disorder derivative from sexual assault during childhood [147], and in diagnosed minors subjected to physical abuse [148].

When it comes to oxytocin, the number of receptors and the blood concentration of this neurotransmitter may vary from one individual to another in response to an array of factors, as well as conditions of stress or distress. It was recently revealed in studies of psychopathology that trauma [149] and severe PTSD [150], as well as major depression disorder in women [151], are linked to extremely increased or fluctuating oxytocin levels, whereas insufficient oxytocin is associated with schizophrenia [152]. It is also known that the way oxytocin responds is partly modulated by the secretion of cortisol, which plays a fundamental role in the biomechanics of stress involving the hypothalamic-pituitary-adrenal axis [153]. However, in individuals diagnosed with PTSD, the system that involves the stress hormone is presumably impaired [154].

Oxytocin dysregulation that foregoes traumatic experiences could amplify the reaction of the central stress response system when dealing with maltreatment and the development of PTSD [155]. Both components mentioned above may work together and increase the cumulative burden of ongoing stress, ultimately triggering and setting the ground for stress-related illnesses.

Besides the aforementioned HPA axis, another stress system is represented by the locus coeruleus (LC) and norepinephrine pathways, which can globally modulate alert and arousal states, as the LC is engaged in times of acute or chronic distress. These two main circuits have been proven to link early-life trauma and PTSD [156].

Therefore, studies that examined the hypothalamic-pituitary-adrenal axis' standard activity in individuals diagnosed with posttraumatic stress disorder have not proven decisive, varying from a lower secretion of cortisol in some cases to a higher one in other cases.

Moreover, another study focused on neurobiological reactions when confronted with white noise and battle cries, where veterans diagnosed with posttraumatic stress disorder displayed increased cortisol amounts in comparison to the control group (of those who served in the military but had no prior diagnosis of PTSD) and controls from the general population [157]. However, there is one thing to ponder: the cortisol evaluation period (a single time prior the experiment and another time postexposure) has been improper to determine standard extent and cortisol response to distress. Therefore, it was deemed inconclusive if the high levels illustrated an increased arousal response or an anticipative reaction induced by the battle sound effects and white noise.

Furthermore, research on animal models reveals that elevated levels of glucocorticoid hormones can cast a shadow on memory functions, as prolonged distress impairs performance and cognitive capabilities by inducing a certain degree of vulnerability and susceptibility to the hippocampus (mediated by glucocorticoid receptors (GCR)) [158].

There are reasons to believe that individuals suffering from stress disorder report a heightened receptiveness of GCR in the hippocampal area [159]. If this is the case, exposure to stress might result in profound deterioration of explicit memory (episodic and sematic) in comparison to controls. That would eventually justify the fact that people diagnosed with stress disorder display impaired cognition and mind tasks, such as difficulties in acquiring fresh information, gaps in the autobiographical memory, and memory loss and dissociative amnesia [160].

In another study, women diagnosed with PTSD displayed remarkably high glucocorticoid amounts in comparison to molested females without the stress disorder when confronted with individualized stories of childhood mistreatment, with the highest peaks occurring in the process and a short time later after being exposed to narratives of

traumatic experience. While recovering, glucocorticoid levels in the posttraumatic stress disorder category became notably low, comparable to those who did not suffer from this disorder [161]. In tune with previous research displaying lowered or adequate basal cortisol amounts, the discoveries can imply there are enhanced cortisol levels after being subjected to distress.

Other interesting findings were revealed in a study conducted by Dr. Young and Dr. Breslau, who focused on PTSD and its biological correlations [162]. It was shown that the category of people diagnosed with this disorder displayed remarkably elevated catecholamine amounts in comparison to the category of individuals subjected to distressing events and the category without any exposure. On the contrary, for mean cortisol levels, there were no significant differences across groups. Women who suffered from major depressive disorder in addition to PTSD displayed remarkably elevated glucocorticoid amounts in comparison to females diagnosed with one or the other psychiatric problem [162].

8. Conclusions

Posttraumatic stress disorder (PTSD) represents a mental health issue that develops after experiencing or witnessing terrifying situations, becoming especially relevant as the ubiquity of this ailment rises with its multifaceted psychosomatic comorbidities. Severe stress, which overcomes adequate response from the patient's side and generates disturbances, perturbations, and pathogenic effects, together with genetics and epigenetics subsequently leads to traumatic stress or psychological trauma.

PTSD is regarded as one of the most complex disorders, influenced by a series of biological, social, and psychological factors. It is also strongly connected to other psychiatric pathologies, such as massive depression disorder, anxiety, impulse control condition, suicide, and drug addiction, while also linked to various physical health diseases and complaints, including neurological, cardiovascular, endocrine, and gastrointestinal ailments.

PTSD aftermath comes forth from a wider network represented by disorders such as the metabolic syndrome, irritable bowel syndrome, bacterial overgrowth syndrome, gut microbiome alterations, hematopoiesis imbalance, or mechanisms/molecules like inflammation, oxidative stress, oxytocin, and cortisol.

Childhood adversities pave the way for psychological problems and psychiatric illnesses later on. Molecules such as cortisol and oxytocin play a significant role in emotional adjustment. Events that profoundly impact one's life during childhood or adolescence can alter and modify oxytocin levels in regions such as the hypothalamus and the amygdala, which are specialized in the production of oxytocin and emotional regulation.

It is wise to have a holistic approach and to perceive this syndrome intertwined with pathologies that arise from the digestive tract, incriminating factors such as the human microbiome and its potential to act as a mastermind inside the body, both as a healer and a disruptor or a trigger to various distresses (such as IBS, obesity, diabetes, and enteral infections). It is noteworthy to observe how the bacterial population impacts general health when it comes to different systems or organs and also that an imbalance of the beneficial/harmful bacteria can tip the scale in favor of a positive or negative outcome.

Equally important is the interwoven nature among metabolic disorders, obesity, or even hematological alterations and PTSD, suggesting there are common underlying neuroendocrine alterations that can enhance the likelihood of a general disturbance of metabolism or just some metabolic changes due to the experienced traumatic event. Processes such as heightened autonomic activity, glucocorticoid activation (by setting a scene for dyslipidemia and weight gain, thus leaning towards a higher risk for metabolic syndrome), immunological dysregulation, and behavioral factors are just a few incriminated links between these disorders.

Furthermore, as stated before, the cerebrum, having an elevated energy demand and requiring 1/5 of total O_2 supply, becomes extremely vulnerable to the oxidation process, especially when it is imbalanced. That essential factor contributes to cerebral biochemical impairment (unrestricted production of ROS, elevated levels of RNS, altered levels of antioxidant glutathione, and reduced production and performance of major antioxidant molecules, thus leading to cellular damage, early instated senescence, and also neurodegeneration). This phenomenon together with an increased level of inflammation (through its proinflammatory cytokines and T regulatory cells) is described in several neuropsychiatric illnesses, and PTSD makes no difference.

In light of current events, the number of individuals diagnosed with posttraumatic stress disorder is envisioned to increase dramatically in the near future, as PTSD represents a serious health matter, which drives the research forward by innovating new techniques, fulfilling the need to further understand this disorder and its underlying mechanisms. In this regard, new developments and improved treatment intervention techniques are expected to benefit the scientific society and, more so, the sufferers, their close ones, and the general population altogether.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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Review Article

The Effect of Bariatric Surgery on Circulating Levels of Oxidized Low-Density Lipoproteins Is Apparently Independent of Changes in Body Mass Index: A Systematic Review and Meta-Analysis

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Background. Obesity is related to dyslipidemia and increased circulating oxidated LDL (ox-LDL) concentrations that may predispose to atherosclerosis. Bariatric surgery may lower the risk of cardiovascular mortality. Elevated plasma ox-LDL has been associated with atherogenesis and atherosclerotic cardiovascular disease (ASCVD) events. The aim of this meta-analysis was to investigate the impact of bariatric surgery on proatherogenic circulating ox-LDL levels in patients with severe obesity. *Methods*. Four databases were systematically searched from inception to May 1, 2021. Also, to clarify the heterogeneity of studies with regard to treatment duration, research design, and the demographic features, a random-effects model and the generic inverse variance weighting approach were utilized. To determine the association with the estimated effect size, a random-effect meta-regression approach was performed. Finally, a meta-regression analysis was conducted to explore the influence of, respectively, baseline and changes in body mass index (BMI), baseline ox-LDL, and postsurgery follow-up period with the estimated effect size of surgery on ox-LDL levels. *Results*. Meta-analysis of 11 studies including 470 subjects showed a significant decline in circulating ox-LDL following bariatric surgery (SMD: -0.971, 95% CI: -1.317, -0.626, $p < 0.001$, I^2: 89.43%). The results of meta-regression did not show any significant association between the changes in ox-LDL after bariatric surgery and baseline BMI, duration of follow-up or baseline ox-LDL values. However, there was a significant association between ox-LDL alteration and percentage of BMI change. *Conclusion*. Bariatric surgery in patients who had severe obesity causes a decrease of circulating ox-LDL that was apparently dependent in BMI changes.

1. Introduction

Obesity is a major risk factor for impaired glucose tolerance, insulin resistance, and type 2 diabetes mellitus, particularly atherosclerotic cardiovascular disease (ASCVD) [1]. Obesity is associated with atherogenic dyslipidemia, low-grade inflammation, and an overall prothrombotic state [2–4]. Dyslipidemia, and especially elevated plasma low-density lipoprotein (LDL) cholesterol, is a pivotal risk factor for atherosclerosis [5]. It is well established that atherogenesis begins with endothelial dysfunction or damage. When LDL particles rich in cholesterol are present in plasma in larger quantities, they permeate through the altered endothelium into the subendothelial space entering the intima. Once this occurs, the LDL particles are oxidized by reactive oxygen species (ROS) and modified into oxidated LDL (ox-LDL) [6, 7]. Ox-LDL particles are strong ligands for macrophage scavenger receptors (CD36, SR-AI/II, and SR-BI) allowing them to enter macrophages and transform them into foam cells [8]. Foam cells, when piled up, appear macroscopically as fatty streaks which are an important step towards fibrolipid atherosclerotic plaques build-up. Therefore, it is not surprising that increased circulating ox-LDL levels are linked to clinical ASCVD events [9].

Bariatric surgery is a durable and effective therapeutic approach in severely obese individuals. Most endocrinology societies recommend surgical therapy for individuals with BMI $\geq 40\,kg/m^2$ or for those with a BMI ranging from 35 to $39.9\,kg/m^2$ and comorbidities who may benefit from weight reduction, as well as for severe obese individuals with a BMI $30.0–34.9\,kg/m^2$ and poorly controlled type 2 diabetes mellitus. The most common types of bariatric surgery are sleeve gastrectomy (SG), Roux-en-Y gastric bypass (RYGP), one anastomosis gastric bypass/mini gastric bypass (OAGB/MGB), laparoscopic adjustable gastric band (LAGB), and biliopancreatic diversion/duodenal switch (BPD/DS) [10]. Weight loss following bariatric surgery can lower ASCVD risk as well as ensuing mortality in severely obese individuals [11–15].

Since severe obesity is associated with dyslipidemia and increased LDL oxidation that may predispose to atherosclerosis and its ominous consequences, it would be important to verify if bariatric surgery would reduce oxidative stress.

Therefore, the aim of this systematic review and meta-analysis was to establish the effect of bariatric surgery on levels of circulating ox-LDL.

2. Methods

2.1. Search Strategy. This systematic review and meta-analysis were done based on the 2009 preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines [16]. PubMed, Embase, Scopus, and Web of Science were searched from inception to May 1, 2021, using keywords in abstracts and titles (also in combination with MESH terms) as follows: ("bariatric surgery" OR gastroplast∗ OR "gastric bypass" OR "Roux-en-Y" OR "gastric band" OR "biliopancreatic diversion" OR gastrectom∗ OR "duodenal switch" OR "gastrointestinal diversion" OR gastroenterostom∗ OR "jejunoileal bypass" OR "obesity surgery" OR "weight loss surgery" OR "weight-loss surgery" OR "bariatric procedure" OR "sleeve surgery" OR "metabolic surgery") AND ("oxidized low density lipoprotein" OR "oxidized LDL" OR "OxLDL" OR "ox-LDL" OR "oxidized Low-Density Lipoprotein" OR "minimally modified oxidized-LDL" OR "MM-LDL" OR "MMLDL" OR "malondialdehyde-low density lipoprotein" OR "malondialdehyde low density lipoprotein" OR "MDA-LDL" OR "MDALDL").

2.2. Study Selection. For inclusion, only original peer-reviewed studies written in English language were considered. All forms of bariatric surgery procedures were taken into account. Articles must have reported circulating ox-LDL before and after surgery. The exclusion criteria were only abstracts, letters, case reports, comments, meta-analyses, duplicate studies, animal studies, reviews, non-English papers, studies with no surgical intervention, and studies without outcomes.

2.3. Data Extraction. Following the removal of duplicate research, two independent authors reviewed the abstracts and titles of the remaining papers for inclusion. The whole texts of the applicable studies were collected. When two papers with the same research purpose were published by the same organization and/or authors, the study published more recently with a larger sample size was included. Any differences were discussed by authors. The following data was gathered from studies which were eligible for inclusion: (1) the name of the first author, (2) the year of publication, (3) the type of surgery, (4) the study design, (5) the characteristics of the patients, (6) oxidized LDL levels, and (7) the period of follow-up.

2.4. Quality Assessment. The Newcastle-Ottawa scale (NOS) was performed to evaluate the study quality in this meta-analysis [17]. Three features of each eligible study are taken into account for this scale: (1) the selection of the studied patients (4 items), (2) the comparability of the studied populations (1 item), and (3) the ascertainment of the exposure (3 items) in case-control studies or outcome of interest in cohort studies.

2.5. Quantitative Data Synthesis. Meta-analysis was performed using Comprehensive Meta-Analysis (CMA) V2 software (Biostat, NJ) [18]. Information regarding sample size, means, and standard deviations from each group were extracted to calculate the standardized mean differences (SMDs). SMD was applied since several different assays were utilized to determine plasma ox-LDL levels. Random-effects meta-analysis was used to get overall estimate of effect size. Postoperative mean and SD were used to calculate the final effect size. To clarify the heterogeneity of studies regarding treatment duration, design of study, and the characteristics of the studied populations, a random-effects model (owing to interstudy heterogeneity) and the generic inverse variance weighting method were utilized [16]. Clinical heterogeneity was judged by study locations and recruited populations, methods applied for ox-LDL assay, baseline ox-LDL values, and differences in biochemical parameters among studied populations. Statistical heterogeneity was appraised by I^2

Table 1: Studies measuring ox-LDL included in the meta-analysis.

Author, year, country	Design of study	Follow-up	Type of surgery	Control (*n*)	Outcome ox-LDL methods of ox-LDL assessment	BMI change (%)	Patients' characteristic Age Sex	No. of patients
Carmona-Maurici et al., 2020 [24] Spain	Prospective observational cohort study	6 months 12 months	Laparoscopic RYGB or SG	—	Significant decrease in ox-LDL levels (Mercodia ox-LDL kit)	-32.27	Obese patients with atheromatous plaque 51.8 ± 1.8 years old 18 (F)/14 (M)	32
						-33.91	Obese patients without atheromatous plaque 43.5 ± 1.8 years old 29 (F)/5 (M)	34
Ho et al., 2021 [25]	Prospective, observational study	6 months 12 months	RYGB, SG, or omega loop bypass	Patients seeking weight management [16]	Unchanged (Mercodia ox-LDL kit)	-29.95	Morbid obesity patients 50.1 ± 10 years old -	59
Coimbra et al., 2019 [26]	Observational study	13 months	Laparoscopic adjustable gastric banding (LAGB)	Healthy volunteers [17]	Significant decrease in ox-LDL levels (Mercodia ox-LDL kit)	-11.85	Obese patients 49.03 ± 10.71 years old 18 (F)/2 (M)	20
Gomez-Martin et al., 2018 [27]	Observational study	6 months	SG	Women matched for age and cardiovascular risk (modified Mediterranean diet) [18]	Significant decrease in ox-LDL levels after 12 months in comparison to baseline and control group (Mercodia ox-LDL kit)	-28.83	Obese women 46 ± 9 years old	20
		12 months	Laparoscopic RYGB			-33.82	Obese women 48 ± 8 years old	20
Kelly et al., 2016 [28]	Longitudinal cohort	6 months 12 months	RYGB or vertical SG	—	Significant decrease in ox-LDL levels at 12 months (Mercodia ox-LDL kit)	-32.6	Adolescents with severe obesity 16.5 ± 1.6 years old 10 (M)/29 (F)	39
		3 months 12 months	Laparoscopic RYGB	—	Unchanged (Mercodia ox-LDL kit)	-35.6	Adolescents with severe obesity 16.5 ± 1.6 years old 3 (M)/10 (F)	13
Müller-Stich et al., 2015 [29]	Prospective cohort	6 months 12 months	RYGB	—	Unchanged (OxiSelect MDA-LDL-quantitation kit)	-25	Patients with BMI more than 35 kg/m^2 and insulin-dependent T2DM 58.6 ± 6.1 years old 10 (M)/10 (F)	20
Van der Schueren et al., 2015 [30]	Observational study	4 months 7 years	Laparoscopic RYGB	Lean controls [24]	Significant decrease in ox-LDL levels at 7 years (Mercodia ox-LDL kit)	-26.6	Obese patients 40 ± 14 years old 5 (M)/12 (F)	17
Julve et al., 2014 [31]	Observational study	6 months 12 months	RYGB	—	Significant decrease in ox-LDL levels (Mercodia ox-LDL kit)	—	Obese patients 20-60 years old 15 (F)/6 (M)	21

Table 1: Continued.

Author, year, country	Design of study	Follow-up	Type of surgery	Control (*n*)	Outcome ox-LDL methods of ox-LDL assessment	BMI change (%)	Patients' characteristic Age Sex	No. of patients
Martín-Rodríguez et al., 2014 [32]	Prospective cohort	12 months	Bariatric surgery	Lean Control subjects [30]	Significant decrease in ox-LDL levels (immunodiagnostic system)	-31.37	Obese patients without metabolic syndrome 40 ± 9 years old 19(F)/4(M)	23
						-16.33	Obese patients with metabolic syndrome 42 ± 10 years old 34(F)/5(M)	39
Garrido-Sánchez et al., 2008 [33]	Observational study	7 months	Biliopancreatic diversion, or RYGB	Healthy, nonobese persons [11]	Significant decrease in ox-LDL levels (Mercodia ox-LDL kit)	-33.9	Morbidly obese patients with: Normal fasting glucose 39.5 ± 11 years old 14(F)/7(M)	21
						-30.39	Impaired fasting glucose 44.1 ± 10.6 years old 21(F)/10(M)	31
						-30.74	Type 2 diabetes 44.5 ± 7.4 years old 14(F)/7(M)	21
Uzun et al., 2004 [34]	Observational study	6 months	Open Swedish adjustable gastric band (SAGB)	—	Significant decrease in ox-LDL levels (Mercodia ox-LDL kit)	-24.24	Morbidly obese patients 35.1 ± 13.1 years old 10(F)/10(M)	20
			Laparoscopic SAGB		Significant decrease in ox-LDL levels (Mercodia ox-LDL kit)	-24.27	Morbidly obese patients 34.6 ± 9 years old 11(F)/9(M)	20

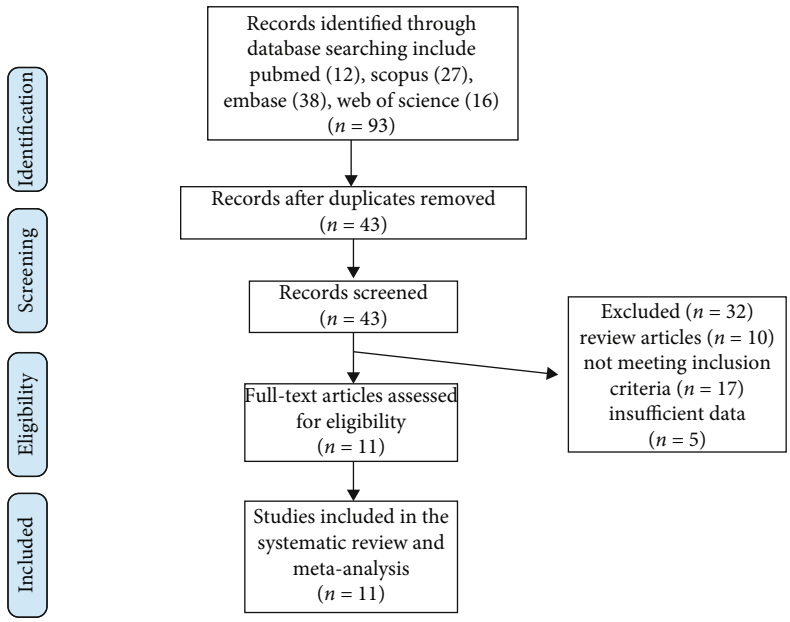
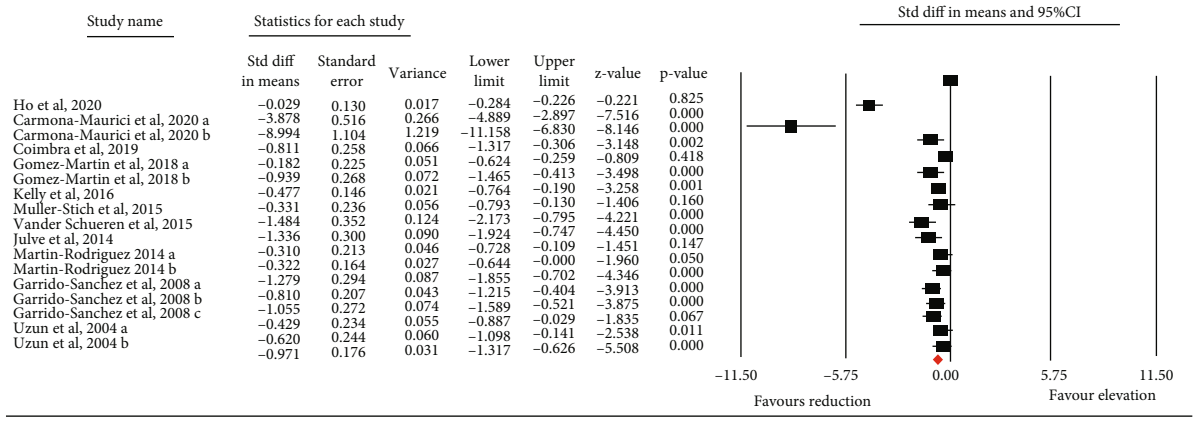


Figure 1: Flow chart of included studies.

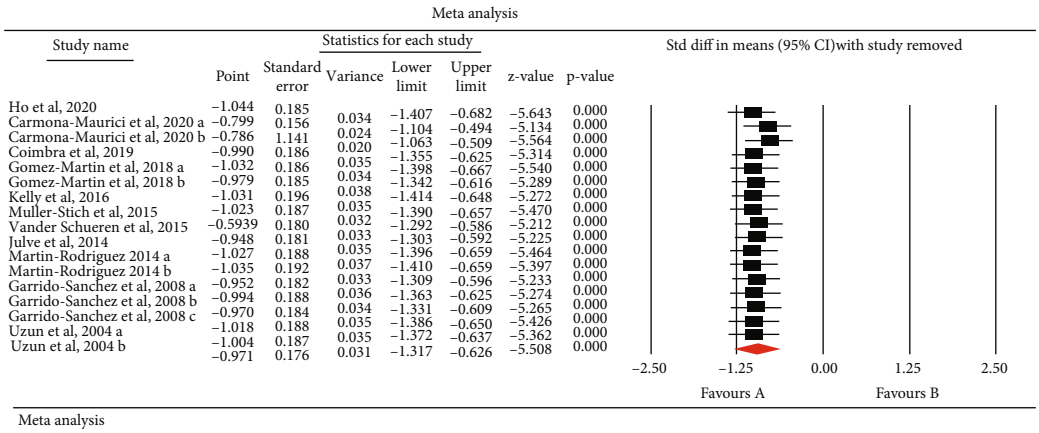
Table 2: Quality of bias assessment of the included papers in accordance with the Newcastle-Ottawa scale.

Study	Selection				Comparability[†]	Exposure		
	Case definition	Representativeness of the cases	Selection of controls	Definition of controls	Comparability of cases and controls	Ascertainment of exposure	Same method of ascertainment	Nonresponse rate
Ho et al. 2021	—	—	—	—	—	*	—	—
Carmona-Maurici et al. 2020	—	*	—	—	—	*	—	—
Coimbra et al. 2019	—	—	—	*	—	*	—	—
Gómez-Martín et al. 2018	—	—	—	—	*	*	—	—
Kelly et al. 2016	—	—	—	—	—	*	—	—
Van der Schueren et al. 2015	—	—	—	*	*	*	—	—
Müller-Stich et al. 2015	—	—	—	—	—	*	—	—
Martín-Rodríguez et al. 2014	—	—	—	—	—	*	—	—
Julve et al. 2014	—	—	—	—	—	*	—	—
Garrido-Sánchez et al. 2008	—	*	—	*	—	*	—	—
Uzun et al. 2004	—	—	—	—	—	*	—	—

[†]Only for comparability a maximum of two stars can be given.



(a)



(b)

Figure 2: (a) Forest plot which displays weighted mean difference and 95% confidence intervals for the influence of bariatric surgery on ox-LDL. (b) Leave-one-out sensitivity analyses for the influence of bariatric surgery on ox-LDL.

index and Cochrane's Q test. The mean and standard deviation were calculated using the method described by Hozo et al. if the outcome measures were reported as median and range (or 95 percent confidence interval (CI)) [19]. When only standard error of the mean (SEM) was supplied, SD was calculated using the following formula: SD = SEM sqrt (n), where "n" denotes the number of participants. A sensitivity analysis using the leave-one-out approach was carried out to explore the impact of each study on the overall effect size (i.e., deleting one study each time and repeating the analysis) [20, 21].

2.6. Meta-Regression. A meta-regression analysis was carried out to investigate the impact of, respectively, baseline and changes in BMI, baseline ox-LDL and duration of postsurgery follow-up with the estimated effect size of surgery on ox-LDL concentrations.

2.7. Subgroup Analysis. A subgroup analysis was conducted to investigate the impact of follow-up duration (≥12 months and <12 months) with the estimated effect size of surgery on ox-LDL concentrations.

2.8. GRADE Scoring. We used the grade of recommendations, assessment, development, and evaluation (GRADE) approach to assess the strength of evidence for each outcome [22]. To summarize the findings for each outcome, the GRADEpro GDT software was used. We assigned four points to each outcome and then evaluated factors that reduced the quality of the evidence. For each outcome, points were reduced based on the presence of the following; the overall RoB for each study, inconsistency (significant heterogeneity), indirectness (significant differences in the population, comparisons, and outcomes), and imprecision (the size of the cohort, width, and significance of the confidence intervals (CIs)). As a result, we classified the evidence into four groups depending on the aggregate GRADE ratings for each intervention: high-grade evidence (at least 4 points), moderate grade evidence (3 points), low-grade evidence (2 points), and very low-grade evidence (1 point).

2.9. Publication Bias. In the meta-analysis, the funnel plot was used to realize the presence of publication bias. Hence, Begg's rank correlation and Egger's weighted regression tests were often performed to help publication bias detection. When asymmetry in the funnel plot was found, potentially

Table 3: Grade assessment.

Summary of findings:					
Effect of bariatric surgery on circulating levels of oxidized low-density lipoproteins in obese patients					
Patient or population: obese patients Setting: - Intervention: bariatric surgery Comparison: -					
Outcome no. of participants (studies)	Relative effect (95% CI)	Anticipated absolute effects (95% CI)		Certainty	What happens
			Difference		
Ox_LDL levels (ox-LDL) assessed with: ELISA/Mercodia/immunodiagnostic/OxiSelect follow-up: range 6 months to 7 years no. of participants: 470 (11 observational studies)	—	The mean ox-LDL levels were 0	— 0 (0 to 0)	⨁⨁◯◯ Low[a,b,c]	
∗The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI). CI: confidence interval					
GRADE working group grades of evidence High certainty: we are very confident that the true effect lies close to that of the estimate of the effect. Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different. Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect. Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.					

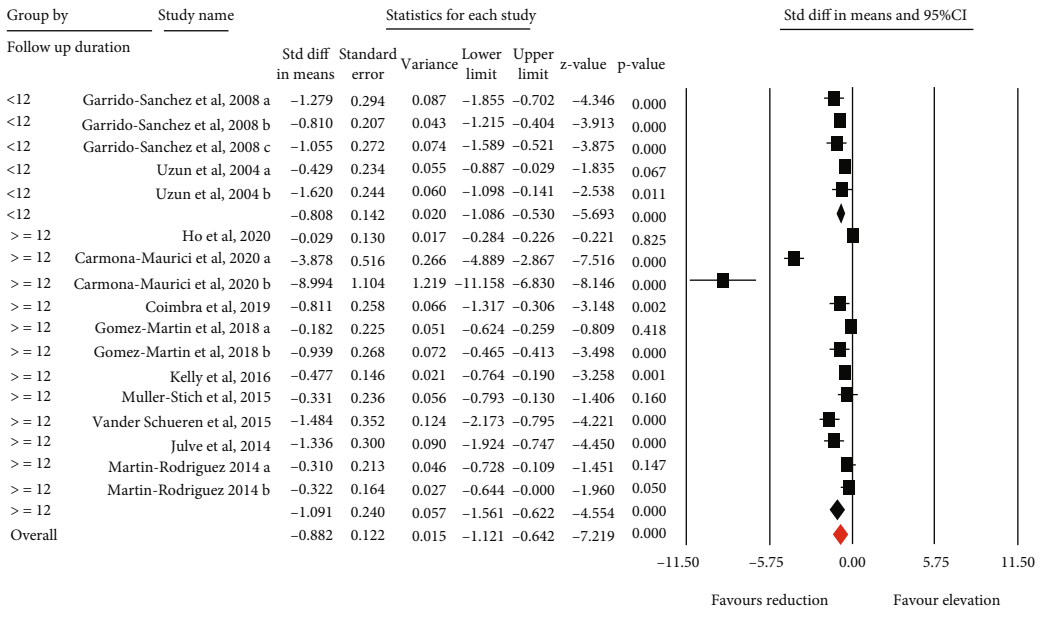
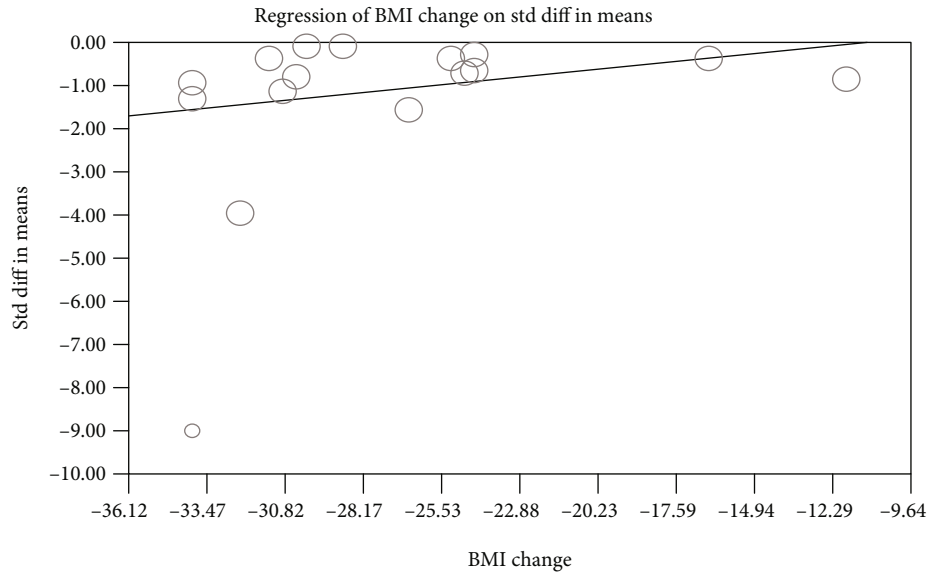


Figure 3: Subgroup analysis to assess the influence follow up duration in ox-LDL alteration.

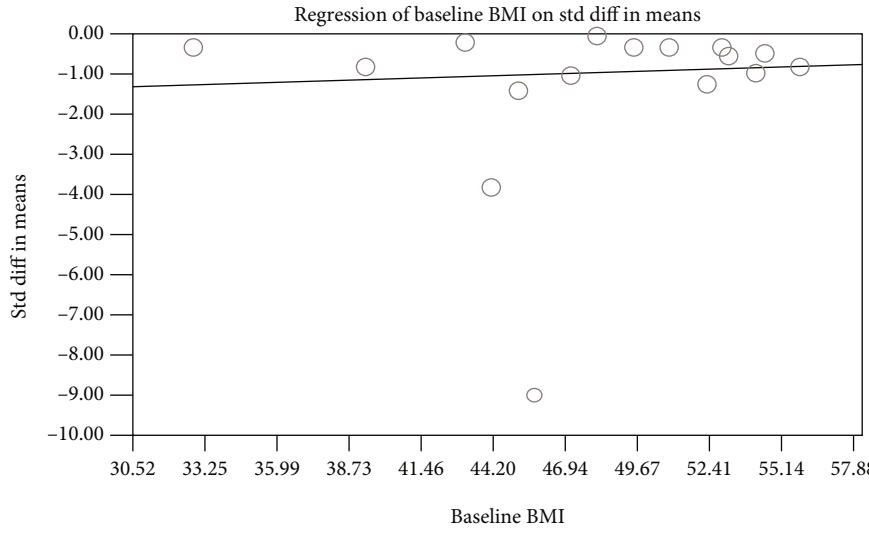
missing studies were inserted using the "trim and fill" method. In the event of a significant result, the "fail-safe N" approach was used to compute the number of potentially missing studies required to make the p value nonsignificant. This is another sign of publishing bias [23].

3. Results

A thorough database search identified 93 published papers, 43 of which were directly connected to the issue of this study. After careful consideration, 32 studies were excluded:10



(a)



(b)

Figure 4: Continued.

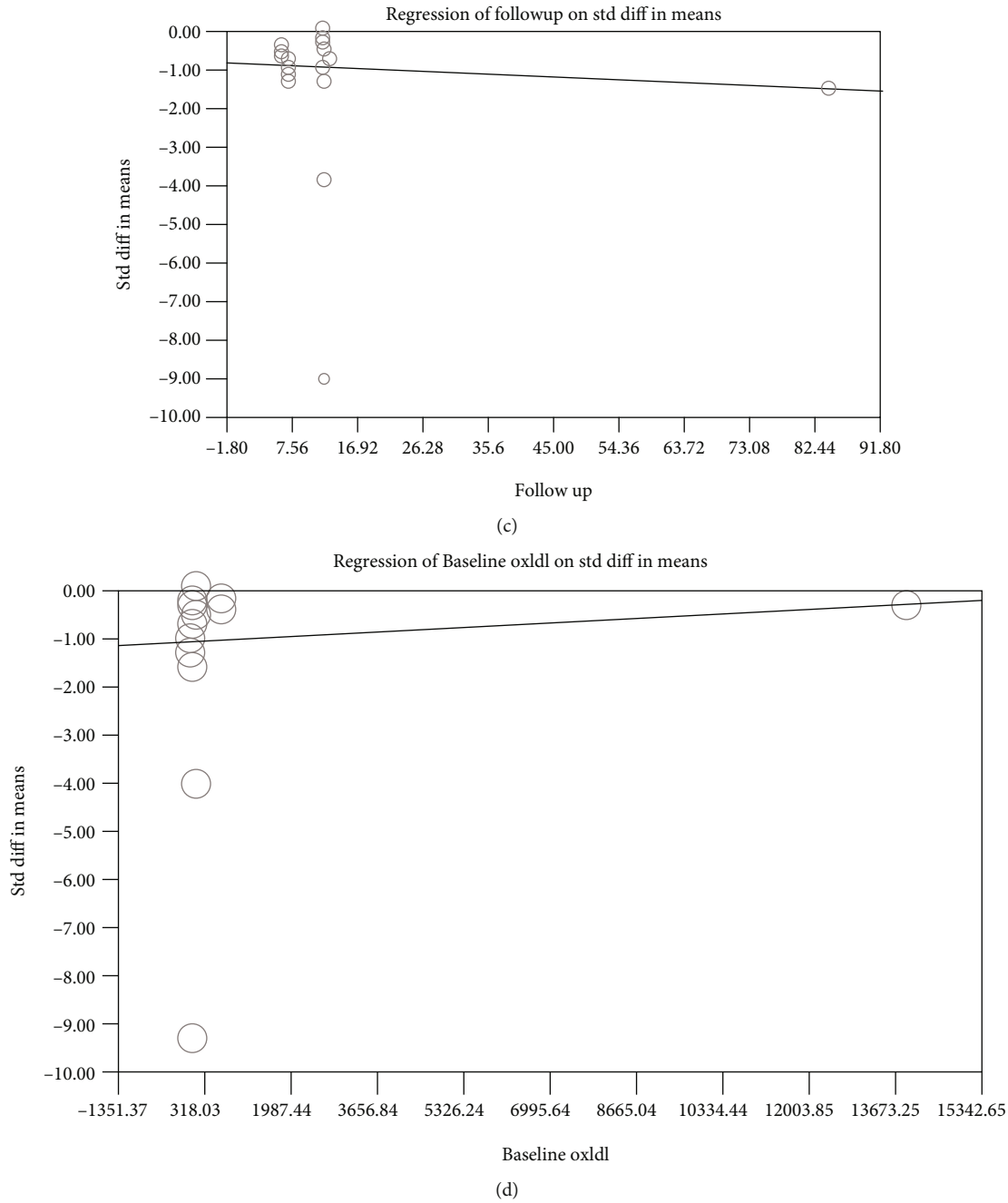


Figure 4: Random-effects meta-regression to assess the influence of % BMI change (a), baseline BMI (b), follow-up duration (c), and baseline oxLDL levels (d) on the estimated effect size.

studies were reviews, 17 studies did not meet the inclusion criteria, and 5 studies did not disclose enough data. As a result, 11 studies which evaluated the levels of ox-LDL after bariatric surgery were included (Table 1). The study selection procedure was indicated in Figure 1.

3.1. Quality Assessment of the Included Studies. All selected studies showed insufficient information for case definition, and most of them had lack of information for representativeness of the cases. Because most of the studies did not include a control group, they were not evaluated for selection of controls, definition of controls, comparability, the same method of ascertainment, and nonresponse rate. However, all studies which included met the ascertainment of exposure criteria. Table 2 shows the details of quality assessment.

3.2. Assay Methods. In most of the included studies, serum ox-LDL was assessed using enzyme-linked immunosorbent assay (ELISA) method. Nine studies used Mercodia ox-LDL kit (Mercodia, Uppsala, Sweden) [24–28, 30, 31, 33, 34], one study used immunodiagnostic system (Boldon, UK) [32], and one study used OxiSelect MDA-LDL-Quantitation kit (Cell Biolabs Inc., San Diego, USA) [29].

3.3. Effect of Bariatric Surgery on Circulating Concentrations of Oxidized LDL. Meta-analysis of 11 publications including 470 subjects demonstrated a significant reduction of circulating ox-LDL following bariatric surgery (SMD: -0.971, 95% CI: -1.317, -0.626, $p < 0.001$, I^2: 89.43%) (Figure 2(a)). In the leave-one-out sensitivity analysis, the reduction in circulating ox-LDL was robust (Figure 2(b)) (low-grade evidence, Table 3).

3.4. Subgroup Analysis. A subgroup analysis was also performed based on follow-up duration (≥12 months and <12 months). Subgroup analyses demonstrated significant reduction of circulating ox-LDL following bariatric surgery in both follow-up periods (≥12 months $p < 0.001$ and <12 months $p < 0.001$). However, this analysis did not show significant associations between follow-up duration and change in ox-LDL levels ($p = 0.309$) (Figure 3).

3.5. Meta-Regression. Random-effects meta-regression was used to assess the effect of potential confounders on the ox-LDL reducing effect of bariatric surgery. The results did not designate any significant association between the changes in ox-LDL and baseline BMI (slope: 0.018; 95% CI: -0.041, 0.078; $p = 0.549$), follow-up duration (slope: -0.007; 95% CI: -0.028, 0.012; $p = 0.444$) or baseline ox-LDL (slope: 0.00005; 95% CI: -0.00005, 0.00016; $p = 0.324$). However, there was a significant association between changes in ox-LDL and percentage of BMI change (slope: 0.069; 95% CI: 0.003, 0.135; $p = 0.039$) (Figures 4(a)–4(d)).

3.6. Publication Bias. Egger's linear regression test (intercept = −7.534, standard error = 0.93; 95%CI = −9.535, -5.533, $t = 8.026$, df = 15, two-tailed $p < 0.001$) and Begg's rank correlation test (Kendall's Tau with continuity correction = −0.801, $z = 4.49$, two-tailed $p < 0.001$) indicated the presence of publication bias in this meta-analysis of bariatric surgery effects on circulating ox-LDL. Trim-and-fill analysis revealed that among all included papers in meta-analysis, there could be five missing studies. The "fail-safe N" test showed that 817 missing studies were required to reduce the effect size to a nonsignificant ($p < 0.001$) value (Figure 5).

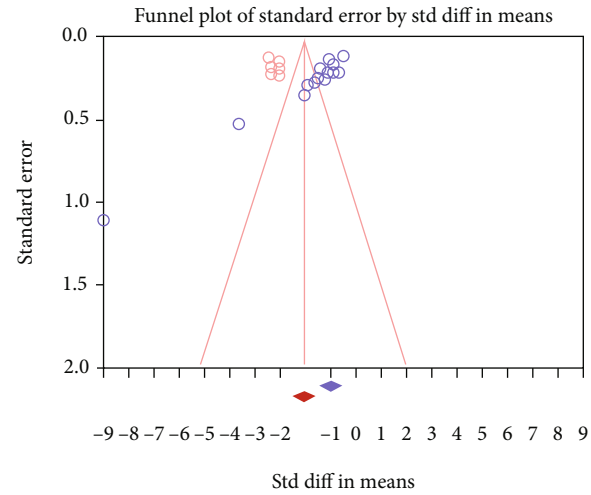


Figure 5: Funnel plot which displays publication bias in the studies reporting the influence of bariatric surgery on oxidized LDL.

4. Discussion

The results of this meta-analysis revealed a substantial decrease of circulating ox-LDL after bariatric surgery. Of importance, the results of meta-regression did not reveal any significant relationship between the changes in baseline BMI, duration of follow-up or baseline ox-LDL value, and ox-LDL after bariatric surgery. However, there was significant association between the changes in ox-LDL after bariatric surgery and percentage of BMI change.

Some authors have tried to explain the beneficial effect of bariatric surgery on ASCVD by decreasing not only body weight, oxysterols, and ox-LDL but also by decreasing plasminogen activator inhibitor-1 (PAI-1), which is elevated in extremely obese patients and by decreased proliferation of vasa vasorum [35]. Bariatric surgery is a well-documented technique for weight loss can have numerous favorable consequences, such as rise in GLP-1 and its potential function in the metabolism including remission of T2DM [36]. Furthermore, bariatric surgery may be a useful therapy for people who have cardiovascular risk factors [37]. Also, bariatric surgery decreases oxidative stress parameters and glycoproteins and increases antioxidant enzymes paraoxonase-1 and catalase as well, which supports the idea that this procedure decreases oxidation of lipoproteins thus having antiatherogenic effects [34, 35].

Others have tried to explain the beneficial effect of bariatric surgery, besides reducing oxysterols and ox-LDL, by increasing not only HDL-cholesterol but also the number of larger HDL particles which are more atheroprotective and reducing the number of small HDL particles which are less protective [9]. Bariatric surgery, apart from decreasing ox-LDL, also reduces the levels of triglycerides and often decreases the number of LDL particles with a decreased proportion of smaller, more atherogenic LDL particles [38]. Already two decades ago, it has been proposed that small dense LDL particles confer greater risk for atherosclerosis and ASCVD than large, buoyant LDL particles, which might be attributed to the greater oxidative vulnerability of small dense LDL [39].

BMI is an indicator of general obesity and demonstrates significant associations with CVDs and ASCVD risk factor. However, as previously suggested, our data support a direct relationship between the magnitude of improvement in cardiovascular risk factors and the amount of BMI reduction [40]. These consequences may be related to positive correlation between change in serum LDL—as a marker of cardiovascular status—and change in BMI [41]. Furthermore, weight loss-induced LDL reduction might decrease the circulating level of the substrate (i.e., LDL particles) for oxidation, and this could partially account for reduction in the generation of ox-LDL [42]. Also, some think that waist circumference, which has been shown to be associated with elevated ASCVD risk, is a better indicator of abdominal obesity [43]. The others suggest that waist-to-hip ratio is even better for measuring abdominal obesity, and it has been proven that this measure corresponds with ASCVD [44].

The favorable effects of bariatric surgery are most likely to be multifactorial [45]. It should be highlighted that weight reduction is not always required to see improvements in some of with ASCVD risks. Indeed, there is considerable evidence to imply that bariatric surgery may cause changes in oxidative stress, inflammation, and adipokines via non-weight loss pathways [28].

Also, this rather surprising issue that bariatric surgery can improve markers of cardiovascular disorders among patients with severe obesity as long as body fat mass is redistributed or replaced by muscle mass independent of significant weight loss needs further investigation.

This study has some advantages. One of them is that this is the first meta-analysis trying to establish the effect of bariatric surgery on circulating ox-LDL in patients with severe obesity. Further investigations should focus on this premise. This meta-analysis has some limitations as well. The studies which were included had an overall relatively small number of patients. Also, there is heterogeneity in types of operations performed, with known differences in metabolic effects between operations. Also, the methods for measuring ox-LDL concentrations in some studies included in this meta-analysis were different and might have explained heterogeneity in our findings. Besides, the quality of the evidence which was evaluated with GRADE approach was low. Additionally, the time intervals and follow-up compliance are not known in some of the reported studies.

In conclusion, bariatric surgery seems to cause a decrease of circulating ox-LDL which is associated with percentage of BMI change, baseline BMI, duration of follow-up, or baseline ox-LDL value. Future studies may focus on the potential neurohormonal effects that could contribute to this reduction, both dependent and independent of weight loss factors.

Data Availability

There is not any raw data associated with this review article.

Additional Points

Key Points. (i) Obesity is related to increased circulating oxidated LDL (ox-LDL) and atherosclerotic risks. (ii) Bariatric surgery may lower the risk of cardiovascular mortality. (iii) Bariatric surgery in severely obese causes a decrease of circulating ox-LDL.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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Research Article

Oxidative Stress Disrupted Prepubertal Rat Testicular Development after Xenotransplantation

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In the past two decades, testicular tissue grafting and xenografting have been well established, with the production of fertilization-competent sperm in some studies. However, few studies have been carried out to observe the development of grafted prepubertal testicular tissue of rats and compare the biological differences between in situ testis and grafted testis. In this study, we established the prepubertal testicular tissue xenografting model using a 22-day-old rat and evaluated certain parameters, including testicular histology, testosterone production, and ultrastructure of the grafted testes. We also assessed gene expression of cell proliferation markers, testicular cell markers, and antioxidative defense system. Our results showed that 47 days after transplantation, intratesticular testosterone concentration was not significantly altered; however, cell proliferation, spermatogenesis, and Sertoli cell markers in the transplanted testes were significantly disrupted compared with the control group, accompanied by aggravated apoptosis and oxidative damage. Moreover, the transplanted testes showed smaller tubular diameter and disrupted spermatogenic epithelium with apparent vacuoles, distorted and degenerated germ cells with obscure nuclear margin, and no spermatids in the center of the tubules. Although testis xenografting has been extensively tested and attained great achievement in other species, the prepubertal rat testicular tissue xenografting to immunodeficient mice exhibited obvious spermatogenesis arrest and oxidative damage. The protocol still needs further optimization, and there are still some unknown factors in prepubertal rat testes transplantation.

1. Introduction

Owing to remarkable progress in the treatment of childhood cancer in recent years, we have seen an increasing number of long-term survivors whose five-year survival rate for all cancers (combined) was 81% in children and 87% in adolescents [1]. Improvement of long-term survival rate is of great significance; however, there is a huge price hidden behind this achievement. Chemotherapy and radiation treatments for cancer can deplete spermatogonial stem cells (SSCs) in the testis, resulting in irreversible infertility [2]. Therefore, impaired fertility is another life crisis that these young individuals must confront, besides cancer itself, and infertility might play an important role in affecting the psychological aspects of their lives [3]. Before undergoing gonadotoxic treatment, adult men can cryopreserve their sperm for future use in assisted reproductive technologies. However, this type of cryopreservation is not an option for prepubertal boys, who are not yet producing sperm at this stage; therefore, preservation of fertility in prepubertal cancer patients has become an important issue [4].

In 1974, Povlsen et al. first transplanted 14- to 22-week-old human fetal organs into nude mice subcutaneously and found fetal testis development after transplantation [5]. However, the study did not attract extensive concern until Honaramooz et al. observed the establishment of complete spermatogenesis after grafting neonatal testis tissue into mouse hosts in 2002 [6]. Moreover, testicular tissue xenografting was used as a model to evaluate gonad toxicity of endocrine-disrupting chemicals and the translation to

humans may offer hope for new strategies to treat male infertility [7]. Mitchell et al. reported that exposure of human fetal testis to di-*n*-butyl phthalate induced no obvious effect on testosterone production by xenografting testicular tissue into castrated male nude mice, which differed greatly from the effect of di-*n*-butyl phthalate exposure on rats [8]. Compared with in vitro fertility preservation methods, testicular transplantation showed advantages of preserving SSCs in the intact testicular niche as well as an established endocrine axis between the host mouse and transplanted tissue, with promising advances towards clinical application [9].

In the past two decades, immature tissue grafting and ectopic xenografting under the skin have been well established. In some studies, when immature testicular tissues from mice, pigs, goats, and monkeys were transplanted under the dorsal skin of immunodeficient nude mice, fertilization-competent sperm was produced and live offspring was generated [6, 10–12]. The status of the donor and the recipient have been proven to influence the outcome of transplantation. Some studies have highlighted the effect of donor age and recipient hormone status on graft survival and development. Compared with immature testicular tissue, the adult testicular tissue transplant usually showed poor outcomes due to its sensitivity to ischemia and hypoxia during the grafting procedure [13]. Different prepubertal donor ages were also proven to affect graft outcome, for example, testicular tissue from a 6-month-old lynx survived better than those from perinatal and 2-year-old lynx after xenografting [14]; therefore, it is still necessary to understand whether prepubertal testes of different stages may exhibit different results after transplantation. Generally, castrated immunodeficient mice were chosen as the transplantation host; however, in some cases, it was found that castration of mice before the transplantation did not modify the outcome of pig testis xenografts [15], and spermatogenic arrest was observed in buffalo testis tissue grafts [16].

Although extensive studies have been carried out to explain the outcome of testicular tissue transplantation, only some have observed the development of grafted prepubertal rat testicular tissue and compared the biological difference between in situ and grafted testes. In this study, we established the prepubertal testicular tissue xenografting model using 22-day-old rats and evaluated parameters including testicular histology, testosterone production, and ultrastructure of the grafted testes. The gene expression of testicular cell markers and antioxidative defense system was also evaluated so as to gain insights into the fertility restoration strategies and the immature testis developmental pattern in different species.

2. Materials and Methods

2.1. Animals and Xenografting. Prior to initiation of the study, the research protocol was reviewed and approved by the Committee on Animal Research and Ethics of Xi'an Jiaotong University (Xi'an, China).

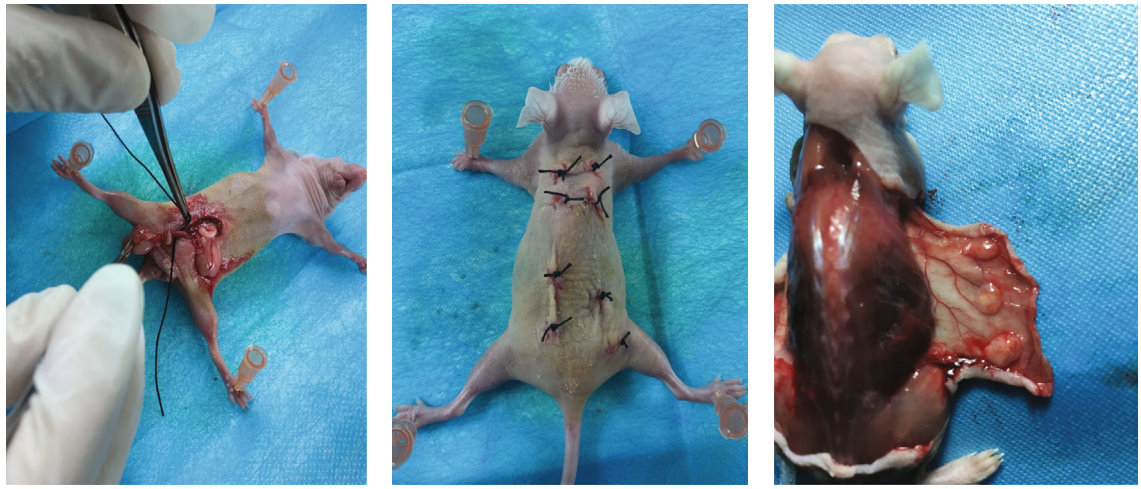
Six specific pathogen-free (SPF) BALB/c male nude mice aged 4–5 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China, and were acclimated for 5 days in Experimental Animal Center of Xi'an Jiaotong University. After acclimation, the nude mice were castrated under anesthesia, and xenograft was performed 2 weeks after castration (Figure 1(a)).

Pregnant SPF Sprague–Dawley rats were obtained from the Experimental Animal Center of Xi'an Jiaotong University. On postnatal day 22 (PND 22), male offspring of these rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital at a dose of 40 mg/kg body weight (Sigma-Aldrich Inc., St. Louis, USA) and then hemicastrated. The left testes were removed aseptically and placed immediately on ice for xenografting. The surgical wound was then sutured, and these male rats were kept as control until PND 69. The left testes that were placed on ice were sliced into small pieces (1–2 mm^3) and transplanted under the dorsal skin of nude mice (Figure 1(b)). Three or four grafts per rat were transplanted to one side of the back of nude mice, and antibiotics were given in drinking water for 3 days. All experimental animals were treated with purified water and food on an ad libitum basis under a 12 h light/dark cycle.

The grafted testes on the dorsal skin of the nude mice were resected on the 47th day after xenotransplantation (Figure 1(c)). On the same day, the right testes of the male rats in the control group were harvested. Part of the tissues was fixed for histology and ultrastructural study, and the rest was frozen under -80°C for gene analysis and intratesticular testosterone analysis.

2.2. Testicular Histology and Staging Spermatogenesis. Following fixation in 4% paraformaldehyde fixative solution at 4°C for 6 h, testicular tissue was transferred to ethanol and xylene, embedded in paraffin, and cut into 5 μm sections. The sections were stained with 0.2% (w/v) hematoxylin for 3 min and 0.5% (w/v) eosin for 6 min and evaluated under light microscopy. Spermatogenesis stages were evaluated after hematoxylin and eosin (H&E) staining and classified into early (stages I–VI), mid (stages VII–VIII), and late (stages IX–XIV) stages. The stages were determined considering certain characteristics, such as changing shape and position of the elongated spermatid in the early stages, the size and position of the residual body for mid stages, and the shape and morphology of the elongating spermatid to identify the late stages. These evaluations were performed by an independent investigator blind to treatment.

2.3. 8-OH-dG Detection. After antigen retrieval and endogenous peroxidase blocking, the sections were incubated at 4°C overnight with anti-8-OH-dG polyclonal antibody (1 : 500, cat# bs-1278R, Beijing Biosynthesis Biotechnology Co., Ltd., China) in a humidified chamber, followed by conjugation to the goat anti-rabbit secondary antibody (cat# SP-0023, Beijing Biosynthesis Biotechnology Co., Ltd., China) and 3,3′-diaminobenzidine (cat# C-0010, Beijing Biosynthesis Biotechnology Co., Ltd., China) staining. The negative control was established with the primary antibody replaced by phosphate-buffered saline (PBS).



(a) (b) (c)

Figure 1: The model of xenotransplantation of testicular tissue: (a) male nude mice were castrated under anesthesia; (b) xenograft was performed 2 weeks after castration; (c) grafted testes were resected on 47th day after xenotransplantation.

2.4. Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay. Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling assay (TUNEL) was performed using a TUNEL Apoptosis Assay Kit (cat# C1098, Beyotime Biotechnology Co., Ltd., China) according to the manufacturer's instructions. In brief, the sections were deparaffinized, hydrated, and incubated with 20 μg/mL DNase-free Proteinase K at 37°C for 20 min. After washing with PBS and incubation with 3% H_2O_2 in PBS at 25°C for 20 min, the sections were incubated with working solution containing TdT enzyme and Biotin-dUTP at 37°C in the dark for 60 min. Next, after washing with PBS, the sections were incubated with streptavidin-horse radish peroxidase solution, followed by DAB working solution. Negative control was set according to the manufacturer's instructions. Seminiferous tubules containing two or more TUNEL-positive cells were counted as positive. The apoptosis index (AI) was calculated as the ratio of number of positive tubules of apoptosis and total number of tubules in a cross section.

2.5. Ultrastructural Study. The harvested tissue were promptly washed with 0.1 mol/L PBS and immersed in 4% (*w*/*v*) formaldehyde and 2.5% (*w*/*v*) glutaraldehyde in 0.1 mol/L PBS for 2 h at 4°C. Then, tissue was postfixed in 1% (*w*/*v*) osmium tetroxide for 2 h in 0.1 mol/L PBS at 4°C for 1 h. After dehydrating, embedding, and sectioning, the sections were double stained with uranyl acetate for 15 min and lead citrate for 5 min. The sections were then observed under an H-7650 transmission electron microscope at 80 kV (Hitachi, Japan).

2.6. Intratesticular Testosterone Analysis. Testicular tissue was weighed and then homogenized in 0.2 mL ice-cold normal saline using an Ultra-Turrax (T8; IKA®-Werke GmbH & Co., KG, Staufen, Germany). Subsequently, testicular homogenates were centrifuged at 3000 rpm for 15 min at 4°C, and then, the supernatant was collected. The intratesticular testosterone concentration was measured using the Elecsys Testosterone II kit (cat# 05200067190, Roche, Germany) according to the manufacturer's instructions. The intratesticular testosterone concentration was expressed as nanogram per gram (ng/g).

2.7. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR). Total RNA was extracted using the TaKaRa MiniBEST Universal RNA Extraction Kit (cat# 9767, Takara, Japan) and converted to cDNA using PrimeScript™ RT Master Mix (cat# RR036A, Takara, Japan). Quantitative real-time PCR was performed using TB Green Premix Ex Taq II (cat# RR820A, Takara, Japan) on the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an endogenous control for normalization. The thermal cycle consisted of initial 2 min at 95°C, followed by 39 cycles of 95°C for 10 s and 60°C for 30 s. All analyses were performed in triplicate samples, and the relative gene expression was analyzed using the $2^{-\Delta Ct}$ algorithm. The names of genes and primer sequences are listed in Table 1.

2.8. Statistical Analysis. Data were expressed as mean ± standard error of mean and analyzed using unpaired two-tailed *t*-test with statistical analysis functions in GraphPad Prism version 8.0 (GraphPad Inc., USA). Differences were considered statistically significant at the probability level of 5% ($P < 0.05$).

3. Results

3.1. Gene Expression of Sertoli Cell Markers. The gene expression of Sertoli cell markers of each group is shown in Figure 2. The expression of Amh in the transplantation group was significantly lower than that in the control group ($P < 0.05$), while the expression of Wt-1 was significantly higher than that in the control group ($P < 0.05$). No

Table 1: The genes and primer sequences.

Gene name	Accession no.	Forward primer	Reverse primer
Gapdh	NM_017008.3	5-GGCACAGTCAAGGCTGAGAATG-3	5-ATGGTGGTGAAGACGCCAGTA-3
Nfe2l2	NM_031789.2	5-ACGTGATGAGGATGGGAAAC-3	5-TATCTGGCTTCTTGCTCTTGG-3
Nox1	NM_053683.1	5-CTCTGCTCCAGAGGAAGAATTT-3	5-CATTGGTGAGTGCTGTTGTTC-3
Nqo1	NM_017000.3	5-GCTGCAGACCTGGTGATATT-3	5-ACATGGTGGCATACGTGTAG-3
Hmox1	NM_012580.2	5-GTCCCTCACAGACAGAGTTTC-3	5-AACTAGTGCTGATCTGGGATTT-3
Sod1	NM_017050.1	5-GGTCCACGAGAAACAAGATGA-3	5-CAATCCCAATCACACCACAAG-3
Sod2	NM_017051.2	5-AGCGTGACTTTGGGTCTTT-3	5-AGCGACCTTGCTCCTTATTG-3
Sod3	NM_012880.1	5-GAGATCTGGATGGAGCTAGGA-3	5-ACCAAGCCTGTGATCTGTG-3
Hsd3b3	NM_001042619.1	5-TTCCTGCTGCGTCCATTT-3	5-GATCTCTCTGAGCTTTCTTGTAGG-3
Lhcgr	NM_012978.1	5-CGCTTCCTCATGTGTAATCTCT-3	5-CCAGTCTATGGCGTGGTTATAG-3
Tspo	NM_012515.2	5-CTATGGTTCCCTTGGGTCTCTA-3	5-AAGCATGAGGTCCACCAAAG-3
Cyp11a1	NM_017286.3	5-AGAACATCCAGGCCAACATC-3	5-CCTTCAAGTTGTGTGCCATTTC-3
Foxa3	NM_017077.2	5-GCTGACCCTGAGTGAAATCTAC-3	5-TCATTGAAGGACAGCGAGTG-3
Amh	NM_012902.1	5-CTAACCCTTCAACCAAGCAAAG-3	5-GGAGTCATCCGCGTGAAA-3
Fshr	NM_199237.1	5-TGTGCCAATCCTTTCCTCTAC-3	5-TGTAAATCTGGGCTTGCATTTC-3
Shbg	|NM_012650.1	5-AAGGACAGAGACTGGACATAGA-3	5-TTAGTGGGAGGTGTGGGTAT-3
Inhbb	NM_080771.1	5-CGAAGGCAACCAGAACCTATT-3	5-TACACCTTGACCCGTACCTT-3
WT-1	NM_031534.2	5-CACCAGGACTCATACAGGTAAA-3	5-TGTTGTGATGGCGGACTAA-3
Dnmt1	|NM_053354.3	5-ACTTTCTCGAGGCCTACAATTC-3	5-TTTCCCTTCCCTTTCCCTTTC-3
Dnmt3a	NM_001003958.1	5-CCACCAGGTCAAACTCCATAAA-3	5-GCCAAACACCCTTTCCATTTC-3
Dnmt3b	NM_001003959.1	5-CGACAACCGTCCATTCTTCT-3	5-GTCGATCATCACTGGGTTACAT-3
Dazl	NM_001109414.1	5-AGTCCAAATGCTGAGACATACA-3	5-TGAACTGGTGAACTCGGATAAG-3
Thy1	NM_012673.2	5-AGAATCCCACAAGCTCCAATAA-3	5-AGCAGCCAGGAAGTGTTT-3
Pou5f1	NM_001009178.2	5-CCCATTTCACCACACTCTACTC-3	5-TCAGTTTGAATGCATGGGAGA-3
Gfra1	NM_012959.1	5-GTGCTCCTATGAAGAACGAGAG-3	5-TGGCTGGCAGTTGGTAAA-3
Boll	NM_001113370.1	5-AACAGCCTGCATATCACTACC-3	5-GCAGATATAGGAATGGAGCAGAA-3
Sycp3	NM_013041.1	5-GAGCCAGAGAATGAAAGCAATC-3	5-GTTCACTTTGTGTGCCAGTAAA-3
Cdc25a	NM_133571.1	5-GTGAACTTGCACATGGAAGAAG-3	5-CTCACAGTGGAACACGACAA-3
Phb	NM_031851.2	5-CATCACACTACGTATCCTCTTCC-3	5-CTTGAGGATCTCTGTGGTGATAG-3
Ldhc	NM_017266.2	5-ATAGGATCCGACTCCGATAAGG-3	5-GCAATGGCCCAAGAGGTATAG-3
Crem	NM_001110860.2	5-GCCAGGTTGTTGTTCAAGATG-3	5-TGTGGCAAAGCAGTAGTAGG-3
Mki67	NM_001271366.1	5-CCGTAGAATTGGCTGGTCTCA-3	5-AGGCTATCAACTTGCTCTGGTT-3
Pcna	NM_022381.3	5-GCCACTCCACTGTCTCCTAC-3	5-CTAGCAACGCCTAAGATCCTTCT-3
Cdkn1a	NM_080782.4	5-CCTAAGCGTACCGTCCAGAG-3	5-GAGAGCAGCAGATCACCAGATTA-3
Cdkn1b	NM_031762.3	5-GATGTAGTGTCCTTTCGGTGAGA-3	5-ACTCCCTGTGGCGATTATTCAA-3

significant difference was found in Shbg, Fshr, and Inhbb expression between the two groups ($P > 0.05$).

3.2. Gene Expression of Leydig Cell Markers and Concentration of Intratesticular Testosterone. The gene expression of Leydig cell markers is shown in Figure 3. The expression of Foxa3 in the transplantation group was significantly lower than that in the control group ($P < 0.05$), while Tspo expression was significantly higher than that in the control group ($P < 0.05$). No significant difference was found in Hsd3β, Lhcgr, and Cyp11a1 expression between the two groups ($P > 0.05$).

The measured intratesticular testosterone concentration is shown in Figure 3. The intratesticular testosterone concentration of the control group (157.07 ± 31.07 ng/g) showed no statistical difference compared with that of the transplantation group (148.40 ± 36.46 ng/g; $P > 0.05$).

3.3. Gene Expression of Mitotic Germ Cell Markers. The expression of mitotic germ cell markers is shown in Figure 4. The expression of Dazl in the transplantation group was significantly lower than that in the control group ($P < 0.05$), while Thy1 expression was significantly higher than that in the control group ($P < 0.05$). No significant difference was found in Gfrα1 and Pou5f1 expression between the two groups ($P > 0.05$).

3.4. Gene Expression of Meiotic Germ Cell and Spermiogenesis Markers. The gene expression of meiotic germ cell markers is

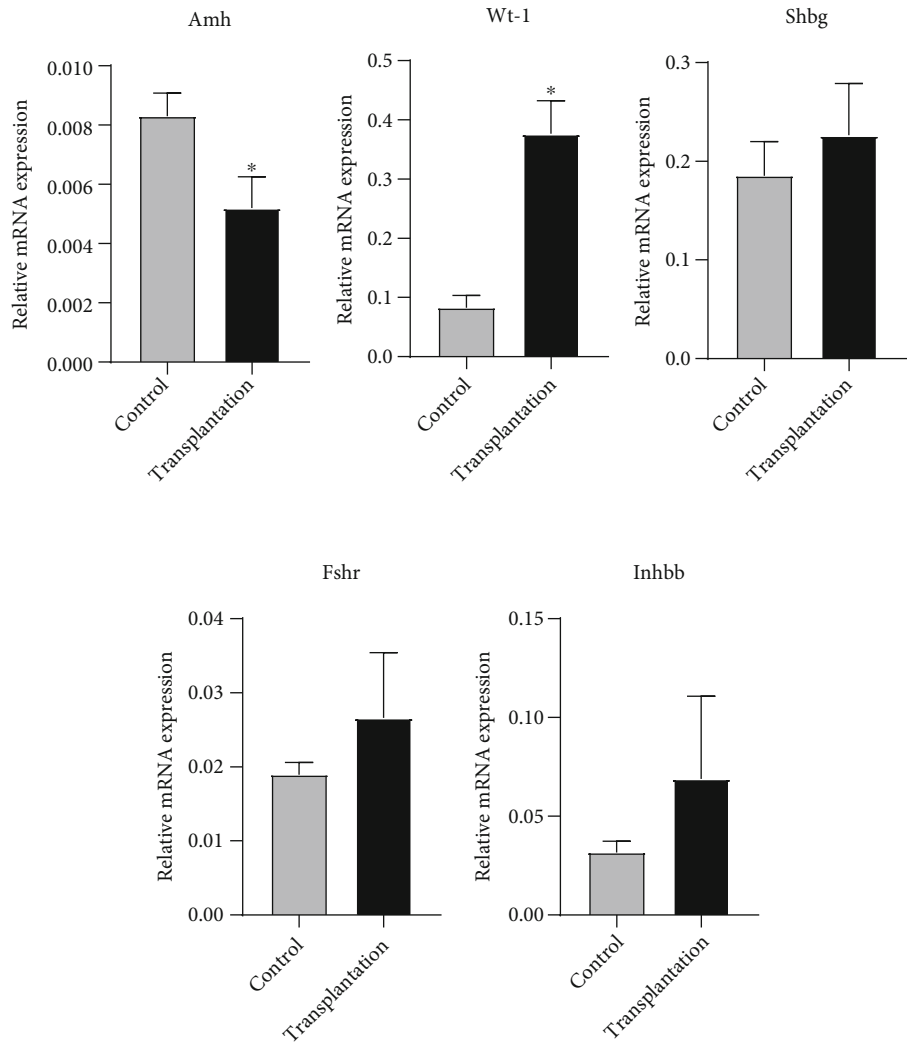


Figure 2: Gene expression of Sertoli cell markers. *Significantly different from control at $P < 0.05$.

shown in Figure 5. The expression of Boll, Sycp3, and Phb in the transplantation group was significantly lower than that in the control group ($P < 0.05$). There was no significant difference in Cdc25a expression between the two groups ($P > 0.05$). In terms of spermiogenesis markers, the Ldhc and Crem expression levels were significantly lower in the transplantation group than in the control group ($P < 0.05$).

3.5. Gene Expression of Methyltransferase. The gene expression of methyltransferase is shown in Figure 6. No significant difference was found in Dnmt1, Dnmt3a, and Dnmt3b expression between the two groups ($P > 0.05$).

3.6. Gene Expression of Antioxidative Genes. The expression of antioxidative genes is shown in Figure 7. The expression of Sod2 and Sod3 in the transplantation group was significantly higher than that in the control group ($P < 0.05$). No significant difference was observed in Nfe212, Nox1, Nqo1, Hmox1, and Sod1 expression between the two groups ($P > 0.05$).

3.7. Gene Expression of Cell Proliferation Markers. The gene expression of cell proliferation markers is shown in Figure 8. The expression of Mki67 and Pcna in the transplantation group was significantly lower than that in the control group ($P < 0.05$). No significant difference was observed in Cdkn1a and Cdkn1b expression between the two groups ($P > 0.05$).

3.8. Testicular Histology. H&E of testicular sections are shown in Figure 9. In the control group, H&E staining showed intact testicular structure without apparent necrosis or vacuoles. Complete spermatogenesis was well established, spermatogonia were seen close to the basement membrane with their dark nuclei, primary spermatocytes were the largest cells, and spermatids appeared smaller than primary spermatocytes and lay near the lumen. By contrast, the transplanted testes showed smaller tubular diameter and disrupted spermatogenic epithelium with apparent vacuoles. The basement membrane was thickened and irregular. Moreover, germ cells in the transplanted testes were loosely arranged, and no spermatids were observed in the center of the tubules. We investigated the stages of spermatogenesis in

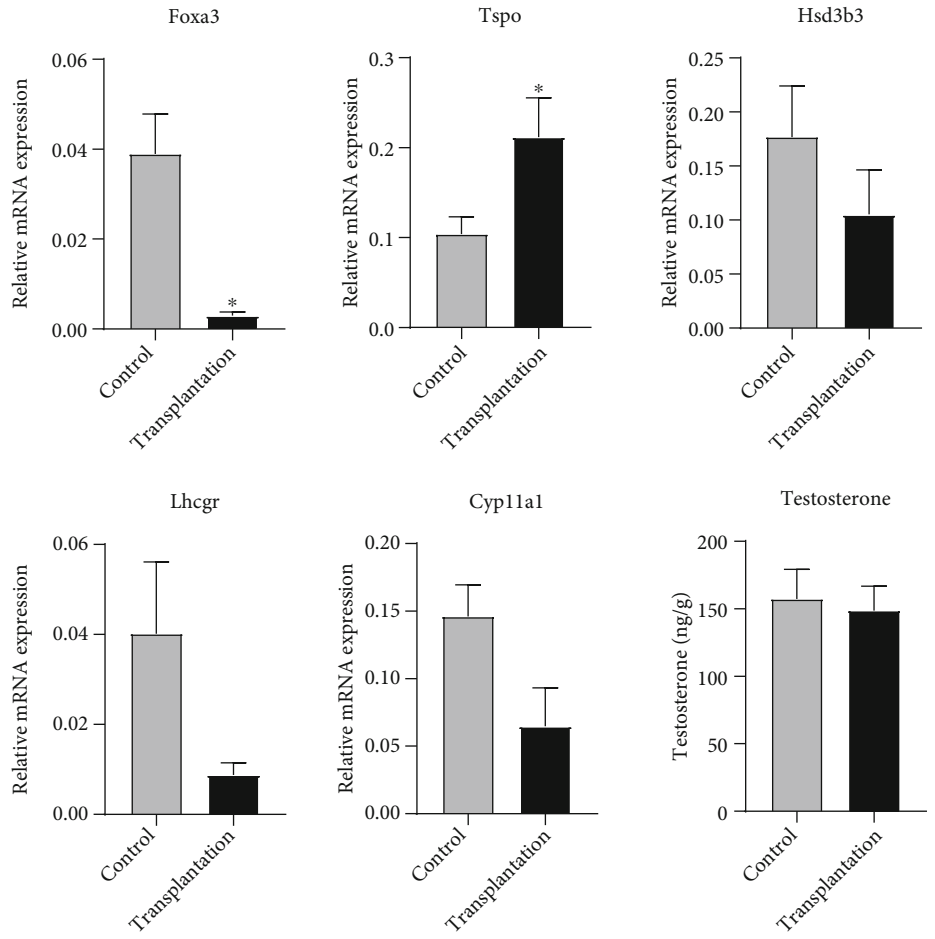


Figure 3: Gene expression of Leydig cell markers and concentration of intratesticular testosterone in control and transplantation groups. *Significantly different from control at $P < 0.05$.

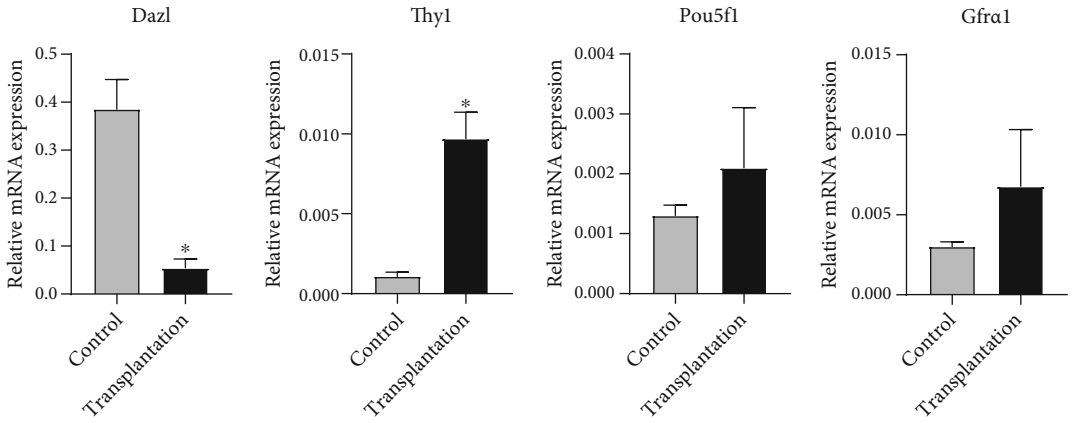


Figure 4: Gene expression of mitotic germ cell markers. *Significantly different from control at $P < 0.05$.

the transplantation and control groups and found that tubules in the grafted testes were all in the late stages, and spermatogenesis stages in controls were normally distributed, indicating that the prepubertal testis transplantation showed deleterious effects on normal testis development, which may lead to adult spermatogenesis arrest.

3.9. Immunohistochemistry of 8-OH-dG. To evaluate the degree of DNA oxidative damage, 8-OH-dG was detected using immunohistochemistry on paraffin sections (Figure 10). In the control group, 8-OH-dG was positive-stained in parts of spermatocytes and interstitial cells. By contrast, testes in the transplantation group were strongly positive-stained in

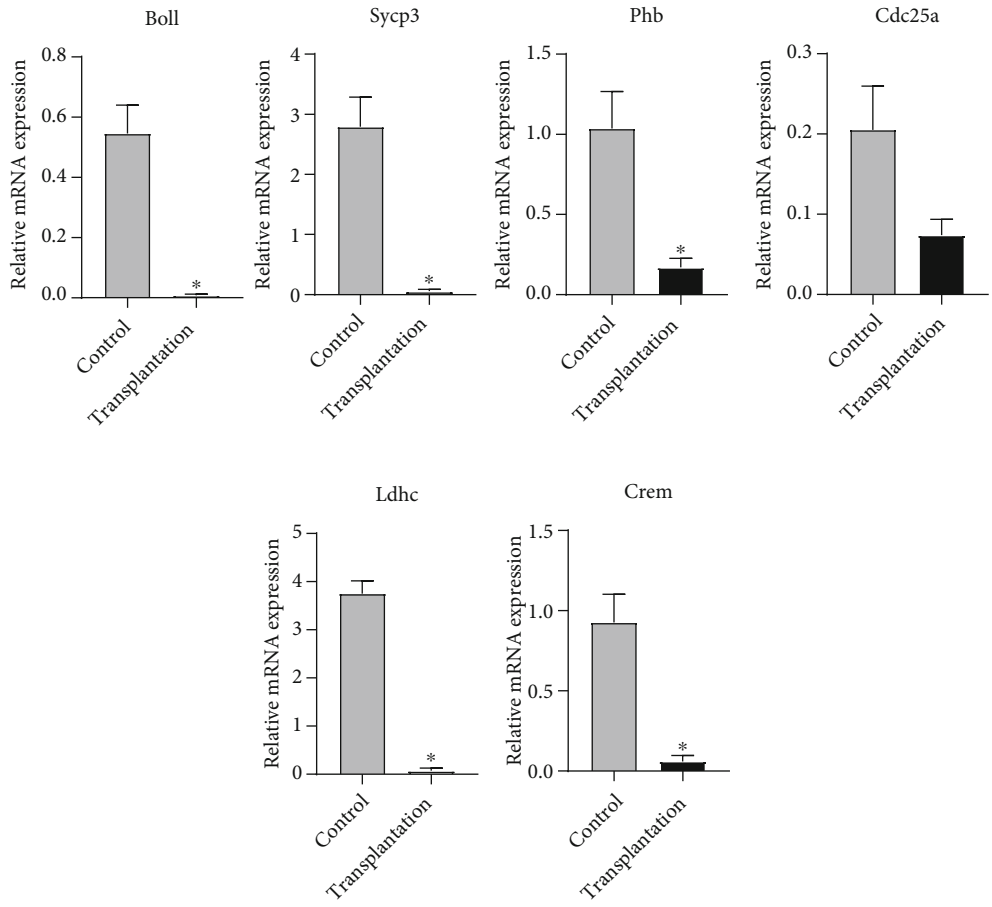


Figure 5: Gene expression of meiotic germ cell (upper row) and spermiogenesis markers (lower row). *Significantly different from the control at $P < 0.05$.

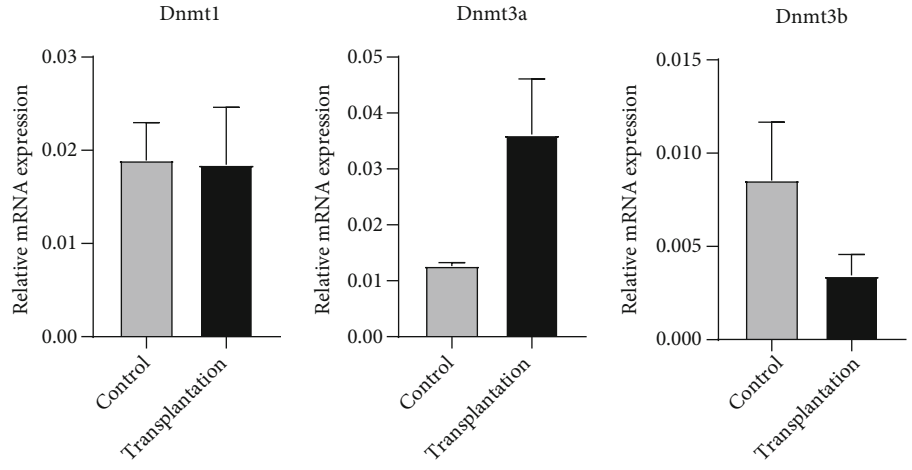


Figure 6: Gene expression of methyltransferase.

the spermatogenic epithelium, and the tubules were deformed and disorderly arranged, indicating that prepubertal rat testis xenotransplantation for 47 days inevitably disrupted the normal spermatogenesis and development of seminiferous tubules, accompanied with aggravated oxidative DNA damage.

3.10. Comparison of TUNEL Assay. The TUNEL assay is shown in Figure 11. The rate of TUNEL-positive cells was generally low in the control group, and the main cell type was spermatogonium. In the transplantation group, more TUNEL-positive germ cells were observed compared with

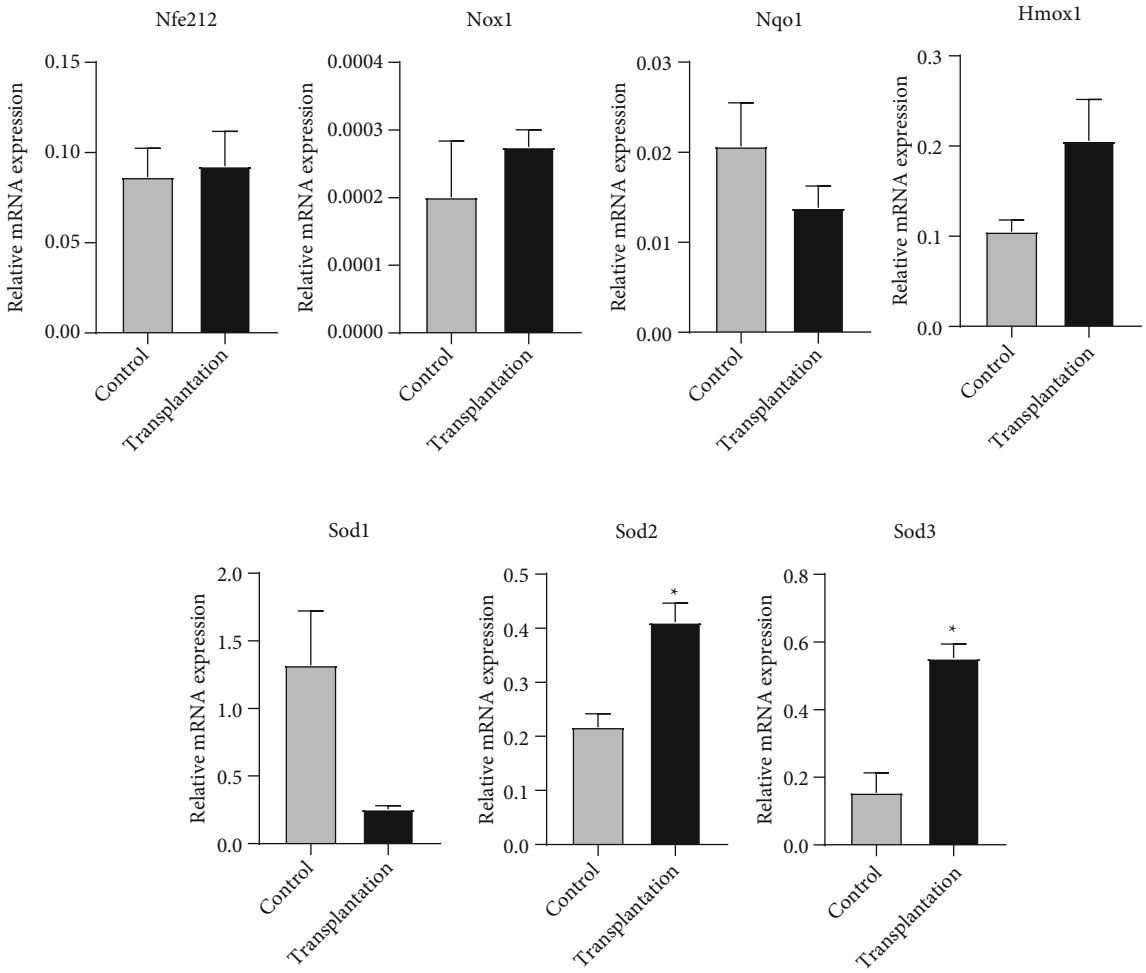


Figure 7: Gene expression of antioxidative genes. *Significantly different from control at $P < 0.05$.

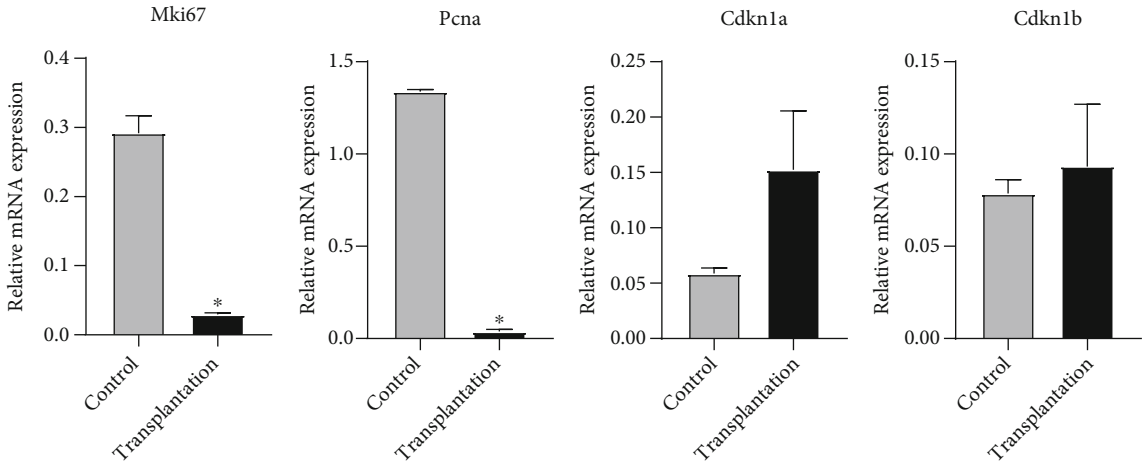


Figure 8: Gene expression of antioxidative genes. *Significantly different from control at $P < 0.05$.

the control group. Moreover, deciduous germ cells were observed in the transplantation group. The irregular seminiferous tubules and AI value in the transplantation group were statistically higher than that in the control group ($P < 0.05$), indicating that oxidative stress may contribute to germ cell apoptosis in the seminiferous epithelium.

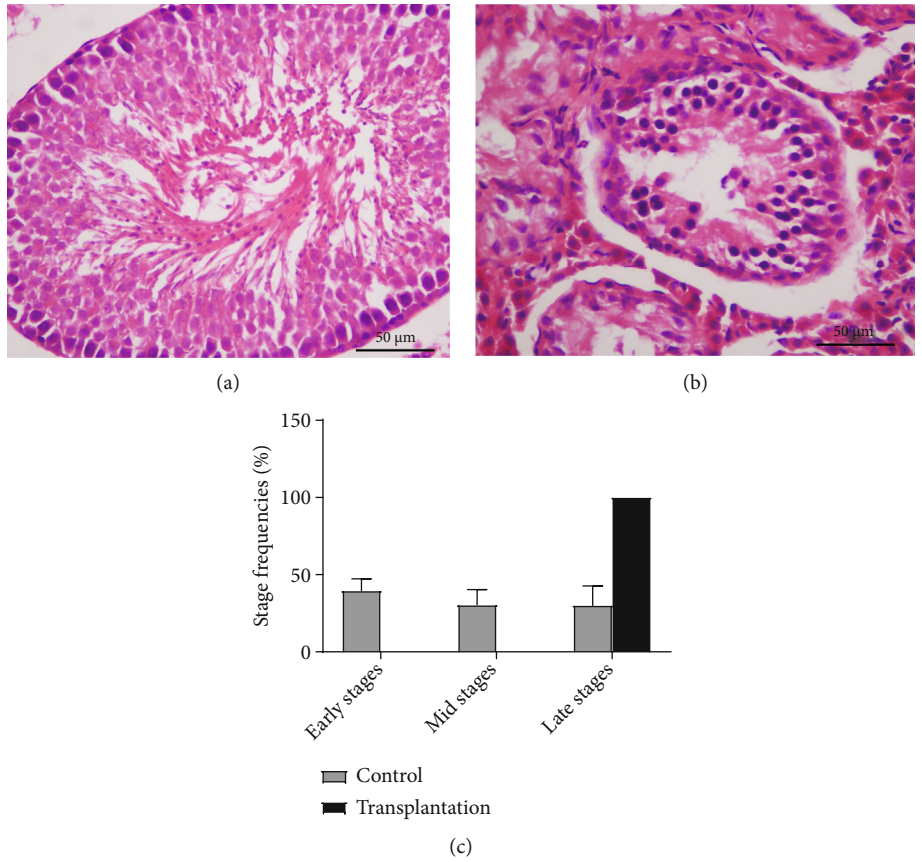


Figure 9: H&E staining of rat testes in control and transplantation groups. H&E staining showed intact testicular structure, and complete spermatogenesis was well established in the control group (a). By contrast, the transplanted testes showed smaller tubular diameter and disrupted spermatogenic epithelium (b). Tubules in the grafted testes were all in the late stages, and spermatogenesis stages in the control group were normally distributed ((c); (a, b): 40x magnification; scale bars indicate 50 μm).

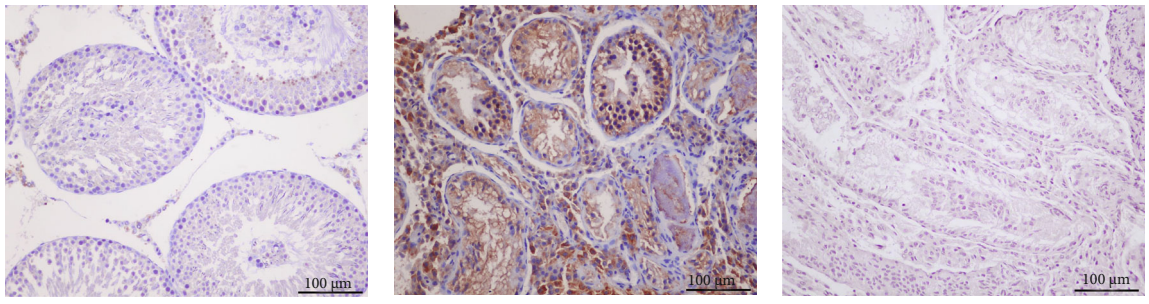


Figure 10: Immunohistochemical staining of 8-OH-dG in (a) control, (b) transplantation, and (c) negative control groups ((a–c): 20x magnification; scale bars indicate 100 μm).

3.11. Ultrastructural Study. The ultrastructure of testis is shown in Figure 12. In the control group, seminiferous tubules were surrounded by an intact basement membrane, and spermatogenic epithelium was well arranged, which was consistent of spermatogonia, spermatocytes, and spermatids. Sertoli cells were identified by their round, but smaller nucleus with weaker electron density, mostly situated near the basal lamina, and the mitochondria were distributed dispersedly in the cytosol. While in the transplantation group, the basement membrane was loosely arranged and the majority of seminiferous tubules showed degenerative changes. Germ cells were distorted and degenerated with obscure nuclear margin, spermatids were rarely seen, and vacuolation was visible in the spermatogenic epithelium. Both groups had abundant mitochondria in Leydig cells, and no obvious swelling was observed in mitochondria and endoplasmic reticulums. Leydig cells in the transplantation group showed higher electron density in comparison with that of the control group.

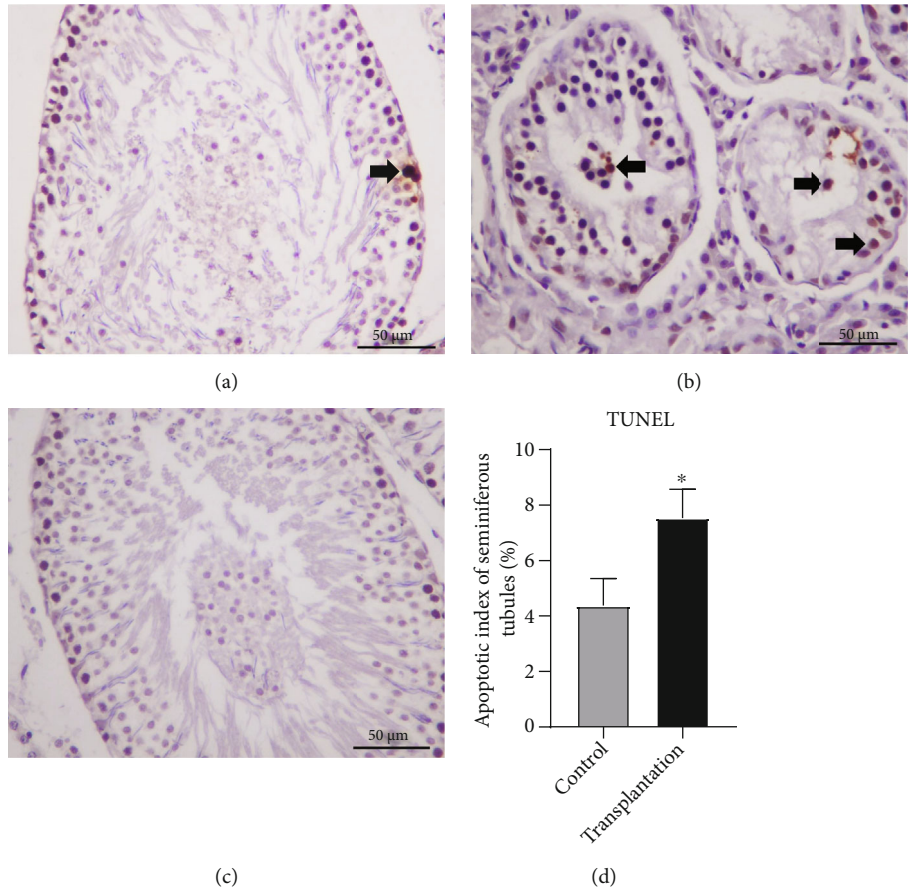


Figure 11: The TUNEL assay in (a) control, (b) transplantation, and (c) negative control groups. The results showed that there were more apoptotic cells (⬆) in the transplantation group than that in the control (d) group ((a, b): 40x magnification; scale bars indicate 50 μm; *significantly different from control at $P < 0.05$).

4. Discussion

With the improvement of cancer therapeutic effects and increase in childhood cancer survival rates, fertility preservation has become an important component of oncologic treatment, especially for those accepting aggressive chemo/radiotherapy [17]. It was found that 46% of all childhood cancer survivors reported infertility, and more than half of the survivors who received alkylating agent chemotherapy had a sperm concentration < 15 million/mL [18, 19]. The disruption of the germ cell population and testicular somatic cells induced by chemotherapeutic drugs/radiation can persist far into adulthood even after treatment cessation. For these reasons, academic societies commonly recommend counseling for pretreatment fertility preservation before the initiation of gonadotoxic therapies [20].

In the past decade, testis tissue grafting has been extensively evaluated in numerous species with variable results; however, many aspects remain unclear due to species difference and complexity of spermatogenesis. In this study, we established the prepubertal rat testicular tissue xenograft model and compared the developmental difference between in situ testis and grafted testis. We found that among Sertoli cell markers, expression of Amh and Wt-1 in the transplantation group was significantly different from that in the control group. Rajpert-De et al. found that the decrease in Amh expression may reflect the terminal differentiation of Sertoli cells and was probably only partially dependent upon a regulatory factor associated with the onset of meiosis [21]. Wt-1 is expressed exclusively by Sertoli cells in the seminiferous epithelium of the adult testis; therefore, Wt-1 knockout resulted in the disruption of developing seminiferous tubules and subsequent progressive loss of Sertoli cells and germ cells. The alternation of Wt-1 in the transplantation group may be responsive upregulation for the maintenance of Sertoli cells and seminiferous tubules in testes [22]. Histologically, disrupted spermatogenic epithelium with apparent vacuoles, along with thickened and irregular basement membrane was observed in the transplantation group. Close and dynamic interactions between germ cells and supporting Sertoli cells are required for the establishment of spermatogenesis. Sertoli cells in the prepubertal period are relatively quiescent, and vacuolation of Sertoli cells is believed to be an early feature of morphological injury, prior to germ cell degeneration [23]. As the tubular vacuoles are usually within or between Sertoli cells, the occurrence of vacuoles is indicative of a breakdown in Sertoli-germ cell junctions and degeneration of germ cells [24].

In this study, we used castrated nude mice as recipients, consistent with previous studies. The removal of the host

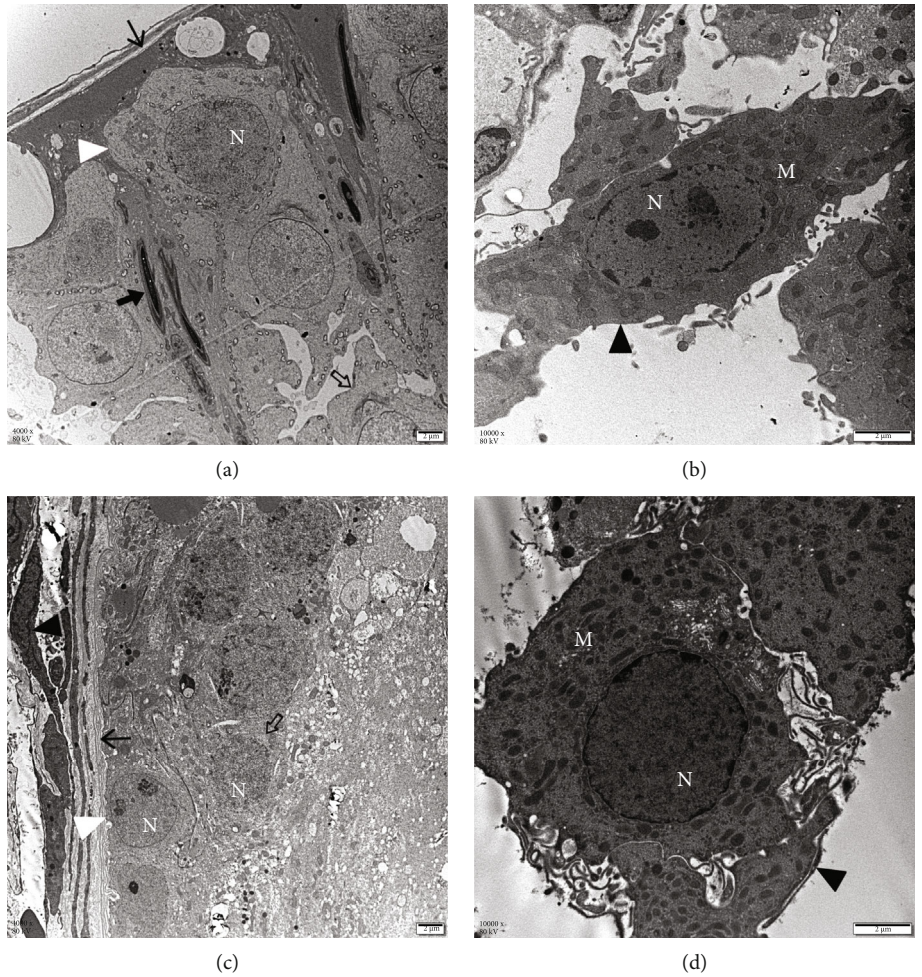


Figure 12: Ultrastructural study of rat testes in (a, b) control and (c, d) transplantation groups (▲: Leydig cell; △: Sertoli cell; ⇧: spermatocyte; ↑: basement membrane; ⬆: elongating spermatids; N: nuclei; M: mitochondria; (a, c): 4000x and (b, d): 10,000x magnification; scale bars indicate 2 μm).

testes can help monitor androgen production by graft Leydig cells and avoid the interference of host testes with xenografts responding to host gonadotropins. Moreover, removal of the host testes released the negative feedback on the mouse pituitary, and a feedback axis would be reestablished between the grafted tissue and the host hypothalamus and pituitary [25]. In this study, we found that the expression of Foxa3 and Tspo expression was significantly altered in the transplantation group. A previous study revealed that Foxa3 was a testis-specific transcription factor, mainly expressed in Leydig cells [26]. Additionally, Foxa3 knockout subsequently induced several gene alterations in mice, including several interesting testis-specific kallikreins implicated in semen liquefaction and male fertility [26]. Tspo is a high-affinity cholesterol-binding protein, which is abundant in Leydig cells and functions as a cholesterol mitochondrial transporter [27]. The differential expression of Foxa3 and Tspo regulated the testosterone production, and the intratesticular testosterone concentration showed no statistical difference between the two groups, indicating that Leydig cell function was less affected and the hypothalamus-pituitary-testis axis was reestablished after transplantation.

Male germ cells are in intimate contact with somatic cells. Of all the cell types, Sertoli cells are located on the basal lamina of the tubules and surround the germ cells by extending elaborate processes. Peritubular myoid cells, located at the extratubular side of the basal lamina, form tubule walls. Spermatogenesis is a highly orchestrated developmental process that can be divided into three parts: spermatocytogenesis, meiosis, and spermiogenesis. In this study, we found that more than half of the mitosis, meiosis, and spermiogenesis markers were significantly downregulated. Moreover, the gene expression of Mki67 and Pcna in the transplantation group was significantly lower than that in the control group, indicating the suppression of testicular cell proliferation in the transplantation group. Among all cell markers, Dazl is mainly expressed in the early stages of spermatogenesis, with highest levels in pachytene spermatocytes. It was confirmed that disruption of Dazl led to spermatogenesis arrest and loss of germ cells [28]. Boll is a member of the DAZ family, and it plays an important role in testicular function, maintenance, and spermatogenesis. Previous studies have revealed that Boll downregulation was associated with the severity of testicular failure, and loss of Boll may cause male infertility [29]. The

other downregulated genes were involved in synaptonemal complex formation (Sycp3), transcriptional regulation (Crem), mitochondrial function regulation (Phb), and sperm motility maintenance (Ldhc), finally leading to testicular degeneration as indicated in the histological findings. Sycp3 is a functional component of the synaptonemal complexes, and it is considered to determine meiotic progression and structural integrity of meiotic chromosomes [30]. Ldhc is testis-specific and plays a vital role for sperm motility by facilitating the conversion of L-lactate and nicotinamide adenine dinucleotide (NAD) to pyruvate and the reduced form of nicotinamide adenine dinucleotide (NADH) [31]. Phb was found negatively correlated with mitochondrial reactive oxidative species (ROS) levels, and loss of Phb in spermatocytes resulted in complete male infertility, associated with apoptosis resulting from mitochondrial morphology and function impairment [32]. Crem is an important component of the cAMP-mediated signaling pathway, which is essential for differentiation of haploid male germ cells, and lack of functional Crem proteins leads to spermiogenesis arrest at the level of round spermatids [33]. In accordance with the gene alternations, we found that prepubertal testis transplantation showed deleterious effects on testis development histologically and ultrastructurally, finally leading to adult spermatogenesis arrest.

Spermatogenesis is a high energy-demanding process, and low levels of oxidative stress are essential for normal testicular function. In the physiological state, testes are equipped with a potent antioxidant system, which protects testes against oxidative injuries [34]. In this study, we found that spermatogenic epithelium staining showed strong positive 8-OH-dG after transplantation. Moreover, more TUNEL-positive germ cells were observed, in addition to deciduous germ cells. The irregular seminiferous tubules indicated that prepubertal rat testis xenotransplantation for 47 days inevitably disrupted the normal spermatogenesis, accompanied with aggravated oxidative damage. Sod2 and Sod3 encode superoxide dismutases, which catalyze the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide, protecting testicular tissue from oxidative injuries. Although the expression of Sod2 and Sod3 in the transplantation group was significantly higher than that in the control group, the imbalance between the generation and elimination of ROS inevitably disrupted the normal cellular functions and aggravated germ cell apoptosis. Normally, relatively poor vascularization of the testes makes intratesticular oxygen tensions lower than the other parts of the reproductive tracts [35]. However, ischemia and hypoxia are inevitable until a functional circulatory connection is established between host and grafts. It was revealed that a circulatory connection was established between graft and subcutaneous blood vessels by a combination of outgrowing small capillaries from the donor tissue and formation of larger vessels by the host [36]. The lack of uniformity in diffusion and new vessel development could be responsible for the asynchronous development and low efficiency of spermatogenesis.

5. Conclusion

In this study, we established the prepubertal rat testis xenografting model and evaluated testicular development after transplantation. Our results revealed that intratesticular testosterone concentration was not significantly altered following transplantation; however, spermatogenesis and Sertoli cell development in the transplanted testes were significantly disrupted, accompanied with aggravated apoptosis and oxidative damage. Although testis xenografting has been extensively tested with great achievement in other species, prepubertal rat testicular tissue xenografting to immunodeficient mice showed obvious oxidative damages and spermatogenesis arrest. The protocol still needs further optimization, and there are still some unknown factors in prepubertal rat testis transplantation, which requires further study.

Data Availability

Data are available upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Effect of Melatonin Administration on Mitochondrial Activity and Oxidative Stress Markers in Patients with Parkinson's Disease

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Mitochondrial dysfunction and oxidative stress are extensively linked to Parkinson's disease (PD) pathogenesis. Melatonin is a pleiotropic molecule with antioxidant and neuroprotective effects. The aim of this study was to evaluate the effect of melatonin on oxidative stress markers, mitochondrial complex 1 activity, and mitochondrial respiratory control ratio in patients with PD. A double-blind, cross-over, placebo-controlled randomized clinical trial study was conducted in 26 patients who received either 25 mg of melatonin or placebo at noon and 30 min before bedtime for three months. At the end of the trial, in patients who received melatonin, we detected a significant diminution of lipoperoxides, nitric oxide metabolites, and carbonyl groups in plasma samples from PD patients compared with the placebo group. Conversely, catalase activity was increased significantly in comparison with the placebo group. Compared with the placebo group, the melatonin group showed significant increases of mitochondrial complex 1 activity and respiratory control ratio. The fluidity of the membranes was similar in the melatonin group and the placebo group at baseline and after three months of treatment. In conclusion, melatonin administration was effective in reducing the levels of oxidative stress markers and restoring the rate of complex I activity and respiratory control ratio without modifying membrane fluidity. This suggests that melatonin could play a role in the treatment of PD.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder of unknown etiology, characterized by the loss of nigrostriatal dopaminergic neurons, which lowers dopamine levels in the striatum and leads to a movement disorder. Mitochondrial dysfunction, increased levels of oxidative stress markers, α-synuclein protein aggregation, and inflammation are extensively linked with PD pathogenesis [1]. In this regard, α-synuclein protein is capable of interacting with mitochondria, which decreases the activity of the mitochondrial enzyme complex I and significantly increases the production of reactive oxygen species. It has been suggested that mitochondrial dysfunction in nigrostriatal neurons is an event that precedes neuronal death [2].

Currently, the use of molecules with antioxidant activity such as melatonin has been proposed for the treatment of PD. Melatonin is a pleiotropic molecule produced in the

pineal gland and other tissues and is involved in multiple physiological functions such as the control of circadian rhythms, anti-inflammatory properties, mitochondrial biogenesis, and energy metabolism, among others [3, 4]. Melatonin performs various antioxidant functions in the neuron, such as a scavenger of free radicals, and has the following characteristics: (a) it can be transported to different tissues in the body; (b) it is a broad-spectrum antioxidant; (c) it is transported across cell membranes; (d) its metabolites still have antioxidant properties [5]. Melatonin is mainly synthesized in the mitochondria and has been shown in animal models to increase mitochondrial activity by increasing the activity of respiratory complexes and ATP synthesis [6]. Previously, we found that melatonin treatment decreases the activity of cyclooxygenase 2, nitric oxide metabolites, and lipoperoxide levels in PD patients [7].

Proton-translocating NADH: quinone oxidoreductase (complex I) is a very large enzyme catalyzing the first step (electron transfer from NADH to coenzyme Q (CoQ)) of the mitochondrial electron transport chain. Interestingly, dysfunctions of complex I are attributed to decreased catalytic activity and/or increased production of reactive oxygen species [8]. This may cause disturbances in the respiratory control ratio (RCR). The RCR is a widely used parameter of mitochondrial function and indicates the coupling between the electron transport system and oxidative phosphorylation. Thus, high RCR indicates good function, and low RCR usually indicates dysfunction [9]. The aim of this work is to study the effect of melatonin supplementation on oxidative stress markers in plasma and mitochondrial activity (particularly, RCR and complex I enzymatic activity) and membrane fluidity in platelets of PD patients. Platelets have been used as a model for neurodegenerative diseases such as schizophrenia, PD, and Alzheimer's disease because evidence has been found that they produce neurotransmitters and contain proteins associated with neurons [10].

2. Materials and Methods

2.1. Study Design. A placebo-controlled, cross-over, randomized, double-blinded clinical trial was performed at the Movement Disorders Clinic of the Neurology Department of the Western National Medical Center, Mexican Institute of Social Security in Guadalajara, Jalisco, Mexico. This study was performed according to the updated Declaration of Helsinki, and all procedures were approved by the Ethics and Health Research Committee of the Mexican Social Security Institute (Protocol number: R-2018-785-019). The selected patients had stages 1–3 PD based on the Hoehn and Yahr scale, were more than 20 years old, and agreed to sign the informed consent letter. Excluded were patients who had movement disorders other than PD, those with previous thalamotomy, pallidotomy, or deep brain stimulation; pregnant females; and use of alcohol, coffee, or any antioxidant supplement. The design of the study has been previously described [11].

Melatonin and placebo were administered in a pharmaceutical gel form packet provided by the company Kurago Biotek®. The pharmaceutical gels were identical in appearance and packaging. Participants reported daily consumption of the supplement in a consumption publication sheet. The researchers were blinded to treatment until the study was complete.

Patients were divided into two groups using random generator software: the melatonin-placebo group and the placebo-melatonin group. The melatonin-placebo group received 25 mg melatonin at noon and 30 minutes before bedtime for three months, followed for four days without treatment (washout period), and then received 25 mg of placebo at noon and 30 min before bed for three months. The placebo-melatonin group received initial placebo during 3 months followed by a washout period and then received melatonin. This melatonin administration dosage and schedule were used in a previous clinical trial of our research group in which the expression of two clock genes (PER1 and BMAL1) were assessed and in which no adverse effects were observed except daytime sleepiness and nighttime problems [11]. Additionally, a control group of thirty clinically healthy individuals was also included to compare the baseline values of the oxidative stress markers and enzymatic activity analyzed in this study.

2.2. Biochemical Assays. Peripheral venous blood was obtained by venipuncture from all study participants after an 8 h overnight fast and collected in Vacutainer® polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing ethylenediaminetetraacetic acid. Blood samples were centrifuged for 10 minutes at 1800 rpm at 4°C. The plasma and erythrocytes were separated immediately. The plasma was centrifuged at 3500 rpm for 15 minutes, and the supernatant was removed. The platelets were resuspended in KME buffer (20 mM (3-(N-morpholino) propanesulfonic acid)) (pH 7.2), 120 mM KCl, and 1 mM ethylene glycol tetraacetic acid (EGTA). Protein determination was carried out by the method of Lowry et al., using bovine serum albumin (BSA) as a standard [12].

Lipoperoxides (malondialdehyde plus 4-hydroxyalkenals) were measured by a colorimetric method using an assay kit (FR12) from Oxford Biomedical Research Inc. (Oxford, MI, USA) following the manufacturer's instructions.

Carbonyl groups in proteins were quantified in plasma using the reaction with 2,4-dinitrophenylhydrazine as described by Levine et al. [13].

Nitric oxide metabolites were determined in plasma according to [14] with minor modifications. Briefly, 400 μL of plasma was added 6 mg of zinc sulfate and vortexed. Then, the samples were centrifuged at 10,000 rpm at 4°C for 10 minutes. To the resultant, supernatant was added 100 μL of vanadium chloride (8 mg/mL). To reduce the NO_3^- to NO_2^-, Griess reagent (comprising 50 μL of 2% sulfanilamide and 50 μL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. Following incubation for 30 minutes at 37°C, the absorbance was read at 540 nm.

Catalase activity was assessed in 1 mL of reaction medium containing 65 μM H_2O_2, 60 mM potassium phosphate buffer (pH 7.4) at 37°C, and 100 μL plasma as described elsewhere [15].

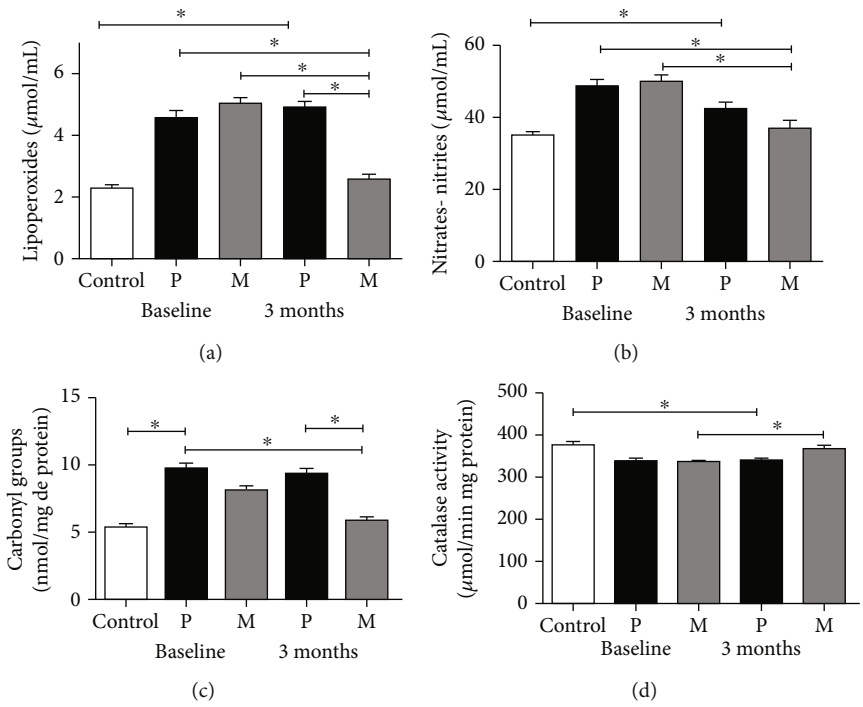

Figure 1: Plasma levels of oxidative stress markers at baseline and after 3 months of treatment in the placebo and melatonin groups. (a) Lipoperoxides (malonaldehyde + 4 hydroxyalkenes), (b) nitric oxide metabolites (nitrates and nitrites), (c) carbonyl groups in proteins, and (d) catalase enzyme activity. Data of the mean ± standard error and a $p < 0.05$ are shown.

For the enzymatic activity of the mitochondrial complex I activity quantification, platelets were lysed by sonic oscillation in a Labsonic U Braun sonicator for 20 seconds and the quantification was carried out as described elsewhere [16]. In brief, 50 μL of samples was incubated at 37°C for 3 min in the reaction medium containing 25 mM of potassium phosphate, 3.5 g/L of BSA, 60 μM of 2,6 dichlorophenolindophenol (DCPIP), 70 μM of decylubiquinone, and 1 μM of antimycin A. Afterwards, 20 μL of a solution containing 10 mM of nicotinamide adenine dinucleotide, 50 μL of BSA (70 g/L), and 5 mM of potassium phosphate (pH 7.4) was added. The absorbance at 600 nm was then recorded every 30 seconds for 5 minutes. Subsequently, rotenone was added and the absorbance was recorded as above. The reduction speed of the DCPIP was determined considering its molar extinction coefficient of 21.3 mM^{-1} cm^{-1}.

Mitochondrial oxygen uptake was measured using a Clark-type O_2 (Oxytherm System, Hansatech Instruments, Norfolk, England) electrode at 30°C in an air-saturated medium as reported previously with minor modifications [17]. The reaction medium (1 mL) contained 130 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1 mM EGTA, 3 mM $MgCl_2$, and 10 mM potassium phosphate (pH 7.4). Respiration in state 3 was measured after the addition of adenosine diphosphate (250 μM) 2 min after preincubating the platelets. State 4 oxygen consumption was determined in the presence of the specific ATP synthase oligomycin inhibitor (8 μg/mg protein). Then, the respiratory control ratio (state 3/state 2) was calculated.

The fluidity of the membranes was determined in platelets via the incorporation of the fluorescent dye 1,3 dipyrylpropane (DiPP) as reported previously. Membrane fluidity was expressed as excimer/monomer fluorescence ratio (Ie/Im), and high Ie/Im ratio indicates high membrane fluidity [18].

2.3. Statistical Analysis. Statistical analysis was performed with the GraphPad Prism v8.0.1 software. Data are expressed as means ± SD. Statistical significance was assessed using the one-way ANOVA test and followed by post hoc multiple comparison tests using Bonferroni correction. Differences were considered statistically significant at p 0.05.

3. Results

A detailed description of the clinical and sociodemographic characteristics of patients included in this study was previously described [11]. No serious adverse drug reactions were observed with melatonin at the doses used during the trial and were mild and transitory. Accordingly, melatonin is a molecule with an uncommonly high safety profile [19, 20].

At baseline, plasma levels of lipoperoxides, nitric oxide metabolites, and carbonyl groups in proteins were significantly higher in PD patients than in the healthy control group (Figures 1(a)–1(c), respectively). Conversely, the plasma activity of catalase was lower in the healthy control group than in PD patients (Figure 1(d)). These data suggest the existence of an active, persistent oxidative stress in PD. After three months of treatment with melatonin, the levels of lipoperoxides, nitric oxide metabolites, and carbonyl groups in proteins were lower than in the placebo group and were statistically similar to the levels of healthy controls.

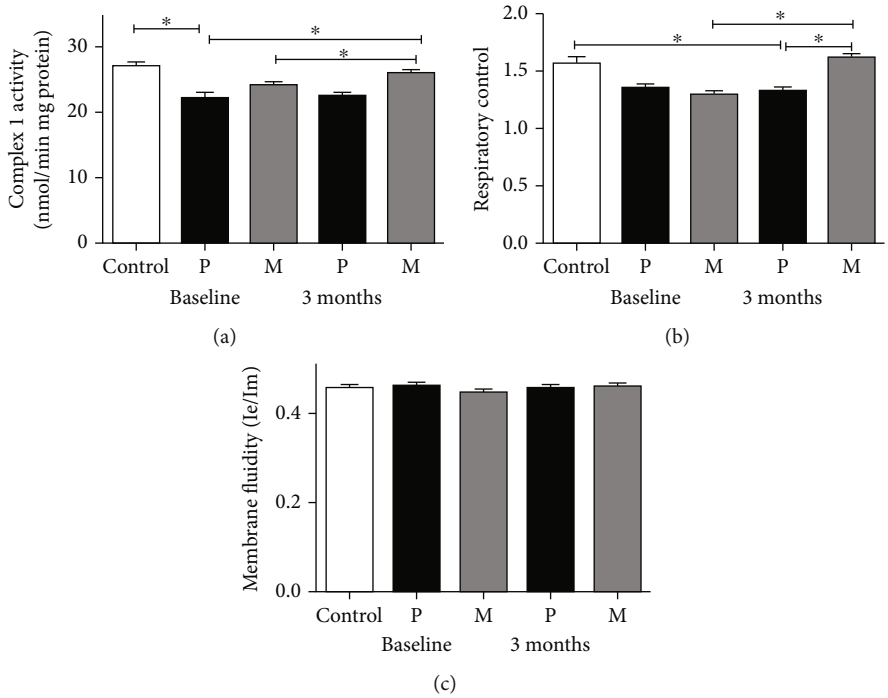

Figure 2: Mitochondrial parameters at baseline and after 3 months of treatment in the placebo and melatonin groups. (a) Mitochondrial complex 1 enzyme activity as measured by the oxidation of NADH, (b) respiratory control ratio, and (c) membrane fluidity. Data of the mean ± standard error and a $p < 0.05$ are shown.

The activity of catalase was increased with the treatment with melatonin at levels similar to the control group.

At baseline, the activity of mitochondrial complex I and the respiratory control ratio were significantly lower in PD patients than in the healthy control group (Figures 2(a) and 2(b), respectively). Compared with the placebo group, the melatonin group showed significant increases of both parameters after 3 months and reached values similar to the healthy control group.

The fluidity of the membranes was similar in the melatonin group and the placebo group at baseline and after three months of treatment and was similar to the control group (Figure 2(c)).

4. Discussion

The results of our double-blind, cross-over trial suggest the existence of an active, persistent oxidative stress status in PD that is linked to lower mitochondrial complex I activity in platelets. These data are in consonance with previously reported data in platelets [21, 22], muscle biopsy [23], and *substantia nigra* [24]. Free radicals are by-products of the mitochondrial respiratory chain and at low concentrations are involved in homeostasis and normal cell signaling. However, increased generation of reactive oxygen species is linked to PD and complex I is one of the main sites of electron leakage to oxygen which leads to the production of the superoxide anion [1, 25]. Furthermore, the assembly of mitochondrial supercomplexes is highly susceptible to oxidative stress. For example, oxidation of phospholipids (particularly, cardiolipin) induces the disaggregation of the supercomplex formed by complex I and complex III, loss of facilitated CoQ channeling, decreased ATP synthesis [26], increased production of reactive oxygen species [27], and favors the release of cytochrome c to cytosol leading to apoptosis [28]. Furthermore, the ratio of reduced CoQ to oxidized CoQ and the ratio of reduced CoQ to total CoQ were decreased significantly in *novo* PD patients [29]. Interestingly, oxidation of cardiolipin in the *substantia nigra* is enhanced by rotenone, an inhibitor of complex I, in a model of PD [30]. Therefore, it can be expected that inhibition of cardiolipin oxidation allows a correct functioning of the mitochondria. Accordingly, as shown in a model of PD, adequate levels of cardiolipin are crucial for efficient electron transport between CoQ and complex [31] and to maintain normal mitochondrial cristae structure and correct assembly of the electron chain supercomplexes [32].

Intervention with daily supplementation of 50 mg of melatonin, for three months, resulted in a significant reduction of oxidative stress markers. These data are according to the reported previously [6] and were paralleled with significant increases of catalase, complex I activity, and respiratory control ratio. In consonance, previous data showed that melatonin increases the levels of reduced glutathione [33], decreases malondialdehyde levels, and stimulates gene expression of important antioxidant enzymes such as superoxide dismutase, complex I, and catalase [34, 35] in rat models of PD. In addition, melatonin prevents cardiolipin loss and oxidation which avoids mitochondrial membrane permeabilization induced by reactive oxygen species and other factors [36]. Reduced glutathione levels are increased by melatonin action, and glutathione also contributes to

maintain the correct mitochondrial redox status and the integrity of the mitochondrial membranes [37]. Melatonin also has anti-inflammatory effects by diminishing cyclooxygenase type 2 activity in PD patients [6] and in MPTP-induced PD in mice [38]. Additionally, melatonin lowers the activation of inducible nitric oxide synthase, a well-known pathological marker of neuroinflammation [39, 40], and also decreases protein lipase A2, lipoxygenase, and cytokine activities owing to its antioxidant actions [41]. Therefore, nitrosative stress and inflammation are diminished by the action of melatonin.

Herein, we find that administration of melatonin is capable of diminishing oxidative stress markers and restoring the enzymatic activity of complex I and the coupling between electron transport and phosphorylation (ATP synthesis) processes (i.e., the RCR). Interestingly, membrane fluidity was not modified by melatonin treatment. Consistent with this proposal, melatonin treatment prevented the loss of the integrity and function of the striatal mitochondria in a chronic model of PD by preserving the normal levels of ATP and mitochondrial respiration [26, 42], and the loss of the mitochondrial membrane potential that may trigger the activation of the permeability transition pore [43]. Furthermore, melatonin significantly decreased neuronal death and mitochondrial fragmentation in an *in vitro* model of PD [44, 45]. Interestingly, it has been proposed that melatonin physically interacts with complex I at its amphipathic ramp close to the site of electron leakage: the iron-sulfur cluster N_2 [46], reverses the decrease in mitochondrial complex 1 activity that is induced by toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [47], and upregulates the expression levels of subunits 1, 3 [48] ND1, ND2, ND4, and ND4L of complex I [49].

Taken together, our data showed that melatonin supplementation recovers mitochondrial function and diminishes oxidative stress. Thus, this indolamine could play a role as an adjuvant in the treatment of PD.

PD is a very complex syndrome, and there are multiple interactions of crucial phenomena such as intracellular mitochondrial dynamics, altered protein degradation, mitochondrial dysfunction, α-synuclein aggregation, calcium homeostasis, and impaired neurotransmitter function. Accordingly to that, a complete molecular map has been proposed that shows all the pathways involved in PD and covers everything from genes, molecules, and cells to metabolic alterations [50]. Considering the above, the limitations of our study were the lack of measurements of the effects of melatonin on some of these phenomena. However, our intention was to evaluate a small part of the mitochondrial defects associated with PD.

Data Availability

Data are available upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Mechanisms of Hydroxyurea-Induced Cellular Senescence: An Oxidative Stress Connection?

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Hydroxyurea (HU) is a water-soluble antiproliferative agent used for decades in neoplastic and nonneoplastic conditions. HU is considered an essential medicine because of its cytoreduction functions. HU is an antimetabolite that inhibits ribonucleotide reductase, which causes a depletion of the deoxyribonucleotide pool and dramatically reduces cell proliferation. The proliferation arrest, depending on drug concentration and exposure, may promote a cellular senescence phenotype associated with cancer cell therapy resistance and inflammation, influencing neighboring cell functions, immunosuppression, and potential cancer relapse. HU can induce cellular senescence in both healthy and transformed cells in vitro, in part, because of increased reactive oxygen species (ROS). Here, we analyze the main molecular mechanisms involved in cytotoxic/genotoxic HU function, the potential to increase intracellular ROS levels, and the principal features of cellular senescence induction. Understanding the mechanisms involved in HU's ability to induce cellular senescence may help to improve current chemotherapy strategies and control undesirable treatment effects in cancer patients and other diseases.

1. Introduction

Hydroxyurea (HU), also called hydroxycarbamide, is a simple hydroxylated compound with the molecular formula $CH_4N_2O_2$, structurally an analog of urea and initially synthesized in 1869 [1–4]. Although HU can exist in two tautomeric forms, the drug primarily adopts the keto form due to its significantly higher stability than the imino form. Moreover, HU is a weak acid containing three ionizable protons, with a pKa of 10.6 [5].

HU is a nonalkylating antineoplastic agent used for hematological malignancies, infectious diseases, and dermatology [6]. The first evidence of its antineoplastic effects was obtained in the late 1950s in experiments conducted on L1210 leukemia cells and solid tumors [7]. In the 1960s, clinical trials demonstrated the drug's efficacy mainly against myeloproliferative disorders [2, 3].

HU has an acceptable short-term toxicity profile in most patients and is currently used as the first-line of chemotherapy in hematological malignancies such as myeloproliferative neoplasm (MPN) characterized by a mutation in Janus kinase 2 (*JAK2*), calreticulin (*CALR*), and myeloproliferative leukemia virus oncogene (MPL) genes [8–11]. Also, this agent is indicated to treat sickle-cell anemia, HIV infection, and thrombocythemia [2, 3, 12]. Moreover, it is effective for the management of refractory psoriasis, likely due to inhibition of epithelial proliferation, thus restoring the typical appearance of the patient's thickened epidermis [13–15]. In addition, HU has been used as a palliative treatment for acute myelogenous leukemia in elderly patients unfit for intensive chemotherapy [16]. Because of its positive effects of therapy, this drug is defined as an "essential medicine" by the World Health Organization [17].

2. Mechanisms of the Inhibition of Cell Proliferation by Hydroxyurea

HU functions as a radiation sensitizer because of its capacity to synchronize cancer cells in the radiation-sensitive cell cycle phase and inhibit the repair response of DNA damage produced by radiation [18]. This drug abolishes the relatively radioresistant cells at the S phase of the cell cycle, reducing highly DNA synthesizing cells and increasing the frequency of the surviving cells at the relatively radiosensitive portion (G1–S interphase) of the cell cycle (Figure 1) [19, 20]. In addition, HU radio-sensitization in patients with advanced cervical cancer increases progression-free survival in the stages III and IVA disease cohort; moreover, HU activities have been evaluated in high-grade gliomas, non-small-cell lung cancer, head and neck cancer, and cervical carcinoma with different grades of success [21].

Furthermore, HU regulates tumor cell resistance to chemotherapy because it accelerates the loss of extrachromosomal amplified genes implicated in therapy sensitivity (Figure 1) [2, 22]. Moreover, it may induce metaphase chromosome fragmentation by directly affecting DNA integrity [23, 24]. The drug cytotoxicity seems to be the result of the DNA damage caused by breaks during DNA synthesis inhibition, which explains its antineoplastic and teratogenic activity. Nonetheless, HU inhibition of DNA replication is reversible, indicating that the drug is likely a cytostatic agent [6]. Indeed, this agent inhibits DNA synthesis in several organisms and in vitro culture cells; thus, it is mainly active in the S phase of the cell cycle, and the reversibility of its action serves as a cell cycle synchronizing agent in cell cultures [25–28].

Mechanistically, the ribonucleotide reductase (RNR), also known as ribonucleoside diphosphate reductase, is a well-established primary cellular target of HU (Figure 1). RNR is an iron-dependent tightly regulated enzyme that catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleotide (dNTP) precursors for de novo DNA replication and DNA repair [29–31]. Three main classes of RNRs have been described according to their metallocofactor requirements. In eukaryotes and eubacteria, class I RNRs are oxygen-dependent and contain a dinuclear metal cluster (Fe or Mn); the other classes II and III are found in aerobic and anaerobic microbes that require a cobalt-containing cobalamin (vitamin B_{12}) cofactor and a $[4Fe\text{-}4S]^{2+/1+}$ cluster coupled to *S*-adenosylmethionine (SAM) for catalytic activity, respectively [32]. Particularly, the mammalian RNR consists of two subunits, α and β, that can associate to form a heterodimeric tetramer, while the human genome encodes one α (RRM1) and two βs (RRM2 and RRM2B) [33]. The α subunit contains binding domains for ribonucleotide substrates (NDPs/NTPs) and allosteric effectors, consequently regulating the RNR complex by nucleotide pools. In contrast, the β subunit possesses catalytic activity and consists of a tyrosyl free radical stabilized by a nonheme iron center necessary for catalysis.

Moreover, the low cell capacity for RNR protein biosynthesis is the rate-limiting step in the de novo synthesis of DNA [30, 34]. Since this enzyme catalyzes the rate-limiting step for DNA biosynthesis, its activity is fine-tuned to generate a periodic fluctuation of dNTP concentration during cell proliferation. In addition, maximum enzyme activity and RRM1 and RRM2 mRNA expression are observed in the S phase of the cell cycle where dNTPs are required [35, 36]. Conversely, at the G_0/G_1 phase, the RNR activity is downregulated due to RRM2 gene transcriptional repression, and in the M cell cycle phase, the β subunit is subjected to degradation pathways by the anaphase-promoting complex Cdh1 binding and consequent polyubiquitination [37, 38].

HU inhibits the RNR activity in vitro and in vivo, and the duration of DNA synthesis inhibition correlates with the level of deoxyribonucleotide pool reduction [39]. For RNR inhibition, HU, due to its small molecule size, penetrates the RRM2 subunit to directly reduce the diferric tyrosyl radical center via a one-electron transfer mechanism [40–44]. Interestingly, the electron transfer from HU to the tyrosyl radical may be mediated by the generation of nitric oxide-like radicals via H_2O_2-dependent peroxidation resulting from the reaction between this agent and the β subunits [44, 45].

Because of the inhibition of RNR enzymatic activity by HU, a reduction of the conversion of ribonucleotides to dNTP occurs, and the consequent dNTP depletion leads to an increase in DNA single-strand breaks [46, 47]. Moreover, the depletion of dNTP pools depends on the exposure length and drug concentration for the treatment [48, 49]. The cell arrest in the S phase due to HU-induced dNTP pool reduction slows down DNA polymerase movement at replication forks, which, in eukaryotes, activates the S-phase checkpoint (also called the replication checkpoint kinase pathway). The S-phase checkpoint is a highly conserved intracellular signaling pathway crucial for the maintenance of genome stability under replication stress. In fact, the S-phase checkpoint preserves the functionality and structure of stalled DNA replication forks and prevents chromosome fragmentation [50–52]. When the S-phase checkpoint is activated, it stimulates RNR activity by increasing RNR β subunit production and regulating its subcellular localization, while the RNR small inhibitor protein expression is downregulated. Furthermore, the activated S-phase checkpoint delays mitosis, suppresses the firing of late origin, and stabilizes the slowed replication forks against collapse, and this allows for the recovery of the regular DNA synthesis rate when the HU effect diminishes [51–54].

Because of low RNR activity, the deprivation of the dNTP pool below the threshold required to sustain DNA replication fork progression may provoke DNA replication fork collapse, which generates strand breaks and oxidative stress. In addition, HU can provoke direct DNA damage at thymine and cytosine residues in vitro, probably because of the Cu(II)-mediated generation of nitric oxide and H_2O_2 [55]. Therefore, these HU's functions may directly cause the permanent effects observed in several cells and discussed later in the text [56, 57].

Even though HU inhibits the RNR activity, which is high in proliferating cells, cells can progress from G_1 to the S phase at a relatively standard rate, where the drug promotes an accumulation of cells at the early S phase. Consequently,

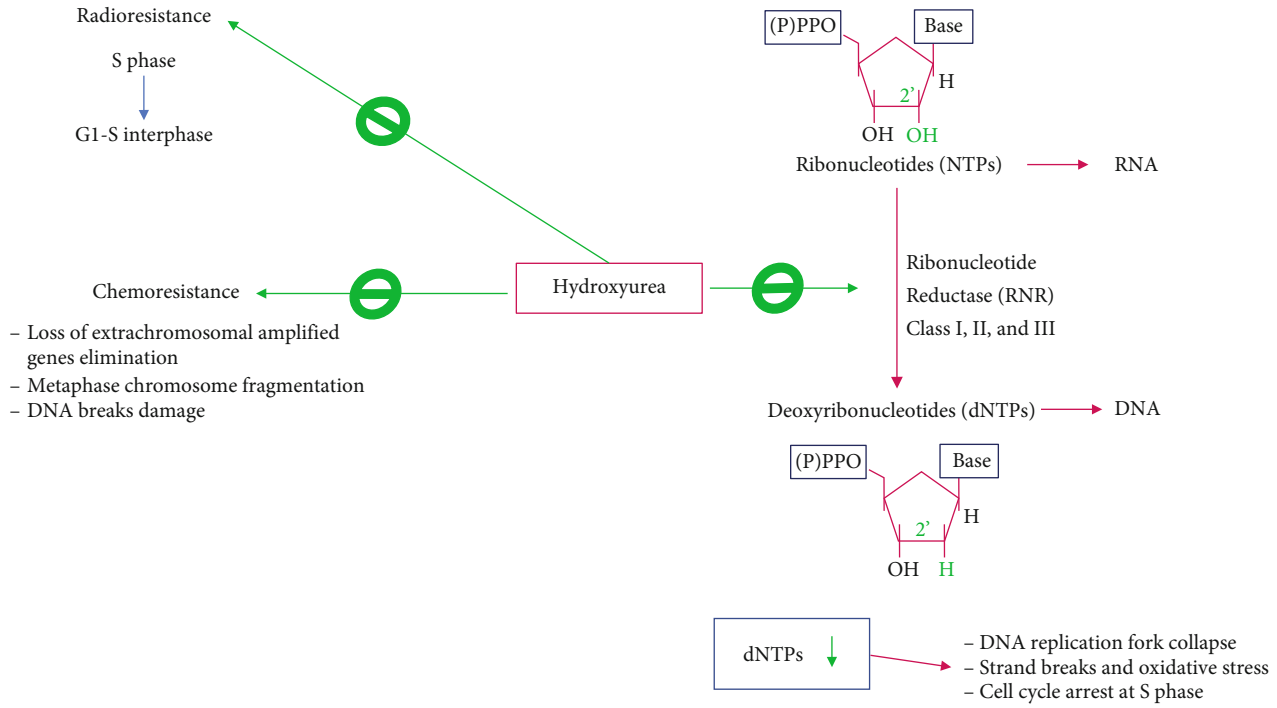

Figure 1: Main mechanisms of hydroxyurea cytotoxicity. HU functions as a radiation sensitizer by synchronizing cancer cells in the radiation-sensitive cell G1-S cycle interphase and inhibition of the DNA damage repair response. Also, HU sensitizes cancer cells to chemotherapy by promoting loss of extrachromosomal amplified gene elimination, metaphase chromosome, and DNA breaks damage. Moreover, HU inhibits the ribonucleotide reductase (RNR) that results in a drastic reduction of the deoxyribonucleotide pool necessary for DNA synthesis. Depletion of dNTPs promotes DNA replication fork collapse, strand break, and oxidative stress. For more details, see the text.

HU reduces the replication fork progression and DNA replication rate [54, 58]. HU selectively eliminates cells in the S phase of highly proliferative cells that are most sensitive to the drug; as mentioned above, HU cytotoxic effects also depend on the dose and duration of exposure [39]. Besides specifically inhibiting RNR, HU also exerts other inhibitory functions on the replitase complex in the S phase of the cell cycle; replitase is a multienzyme complex of mammalian cells that produce dNTPs and deliver them to DNA synthesis by the DNA polymerase. Replitase complex comprises thymidine kinase, dihydrofolate reductase, nucleoside-5′-phosphate kinase, thymidylate synthase, and RNR itself [59, 60].

3. Mechanism of Cellular Senescence

Cellular senescence, defined as a process that causes an irreversible proliferative cell arrest with secretory features in response to several molecular and biological stressors, is a significant contributor to aging and age-related diseases [61–64]. This process was initially described by Hayflick and Moorhead in 1961 [65] when they observed that primary cells undergo a limited number of cell divisions in vitro. This observation allows suggesting a cell-autonomous theory of aging that implies the depletion of active replicative cells required for tissue homeostasis and tissue repair and regenerative processes [62].

Cellular senescence encompasses different biological and molecular events that result in at least three senescence types (Figure 2): In replicative senescence (RS), the main mechanism relies on the number of cellular divisions in culture in vitro and, consequently, telomere shortening due to successive cell duplication [65–68]. Oncogene-induced senescence (OIS) is related to a tumor-suppressive mechanism as a response to oncogene overactivation and overexpression. Oncogenic activation seems to induce a stable growth arrest in premalignant cells from senescence expression, allowing a blockade of genetically unstable cells to progress to dangerous malignant stages. For instance, H-RAS mediates the induction of cell cycle inhibitor $p16^{INK4A}$, which precludes the hyperphosphorylation of RB by the cyclin-D- and CDK4 and suppresses E2F activity. In addition, increased c-Myc expression promotes the $p14^{ARF}$ transcription that stabilizes p53, thus accelerating cellular senescence [69–72]. The cellular senescence induced by oncological agents used at relevant therapeutic concentrations is called chemotherapy-induced senescence (CIS) [73].

In this last context, “immortal” cancer cells can undergo senescence from exposure to chemotherapeutic agents, causing severe cellular stress and displaying both protumorigenic and antitumorigenic functions [74, 75]. The chemotherapeutic armamentarium comprises genotoxic and cytotoxic drugs that target proliferating cells in a variety of cell cycle-dependent mechanisms (Figure 3) [76]. These drugs include topoisomerase inhibitors such as doxorubicin,

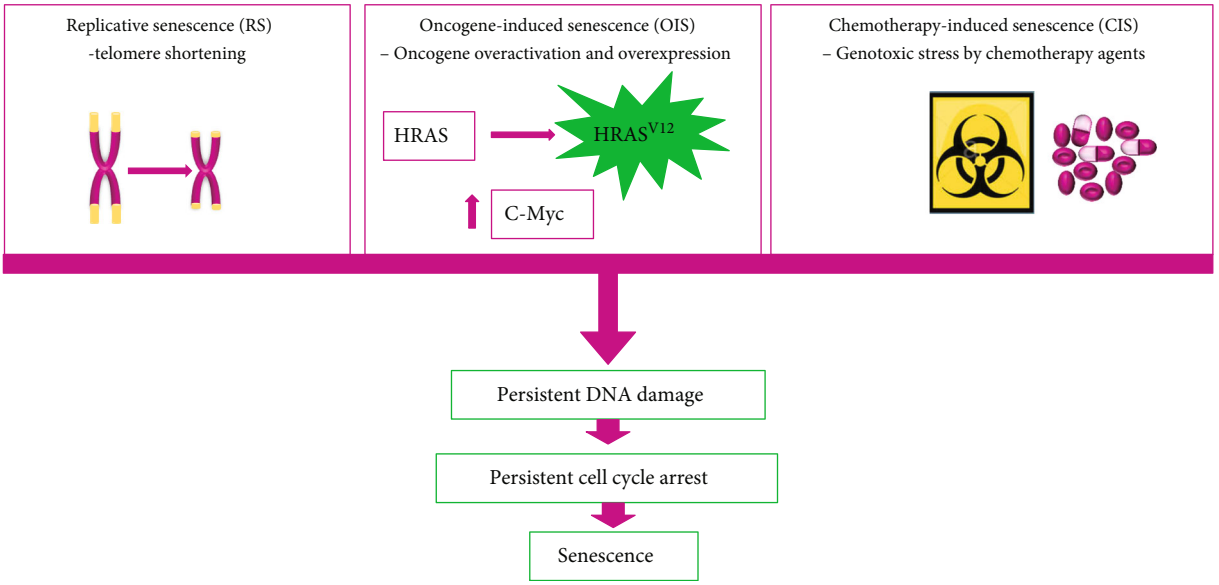

Figure 2: Mechanism of cellular senescence. The figure illustrates the main three senescence types that influence tumorigenesis: replicative senescence (RS) due to telomere shortening from a limited number of cell divisions, oncogene-induced senescence (OIS) due to an aberrant and sustained antiproliferative response to oncogenic signaling resulting from an oncogene-activating mutation and expression or the inactivation of a tumor-suppressor gene, and chemotherapy-induced senescence (CIS) due to cell response to severe genotoxic stress from exposure to a variety of onco-therapeutic agents.

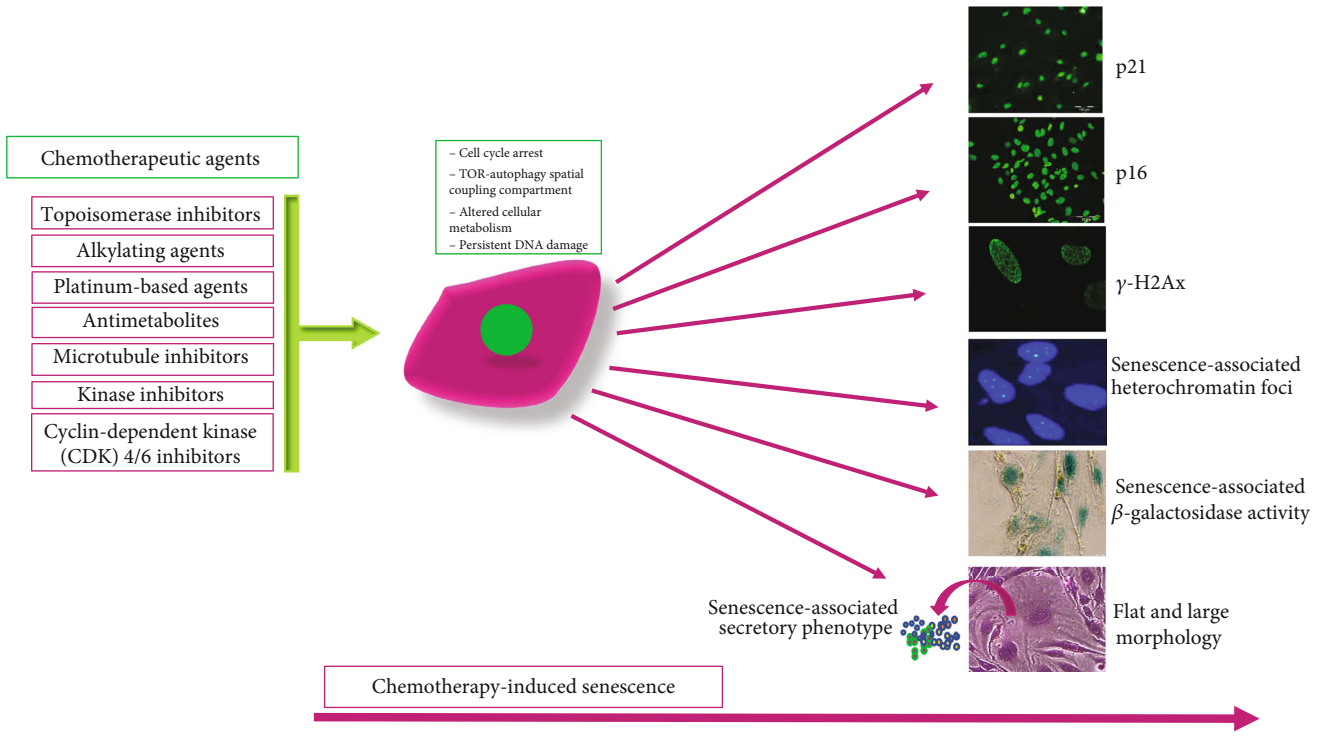

Figure 3: Chemotherapy-induced senescence. The figure indicates the main types of chemotherapeutic drugs with different mechanisms of action that induce genotoxic stress, triggering several cellular and molecular changes that result in the acquisition of senescence phenotype features indicated in the figure, such as increased $p21^{Cip1}$, $p16^{INK4}$, and γ-H2Ax expression, senescence-associated heterochromatin foci formation, expression and activity of senescence-associated β-galactosidase, senescence-associated secretory phenotype, and morphology changes in flat and enlarger cells.

etoposide, and topotecan [77–80]; alkylating agents such as busulfan, cyclophosphamide, and mitomycin C [81–83]; platinum-based agents, including cisplatin, carboplatin, oxaliplatin [84–86]; antimetabolites such as methotrexate, gemcitabine, 5-fluorouracil, and hydroxyurea [87–90]; microtubule inhibitors that comprise paclitaxel, vincristine, and vinblastine [91–93]; kinase inhibitors such as vemurafenib, dasatinib, and lapatinib [94–96]; and cyclin-dependent kinase (CDK) 4/6 inhibitors, including palbociclib, abemaciclib, and ribociclib [97–99].

Interestingly, besides altering cellular cancer states, CIS also affects the tumor microenvironment by acting on noncancerous tissues and promoting immunosurveillance to eliminate tumor cells, while it also may contribute to chronic inflammation and cancer drug resistance [74, 100–102].

With senescence induction, cells display a stable cell cycle arrest and complex phenotypic and molecular changes, such as cell enlargement and flattening, altered cellular metabolism, and dysfunctional mitochondria, and the generation of the cytoplasmic target of rapamycin- (TOR-) autophagy spatial coupling compartment (TASCC) (Figure 3) [103, 104]. Moreover, senescent cells exhibit increased expression and activity of senescence-associated β-galactosidase (SA-β-gal), a lysosomal enzyme that in senescence conditions stains positive at pH 6 and is one of the first characteristic molecular markers for senescence identification (Figure 3) [105]. Furthermore, because of the inherent molecular changes during the display of senescence features, cells suffer persistent damage such as DNA double-strand breaks that triggers a persistent DNA damage response (DDR), resulting in permanent cell cycle arrest [106]. Specifically, DDR is a signaling cascade network that senses and repairs DNA lesions, thus preserving DNA integrity and preventing the generation of undesirable deleterious mutations, which under persistent or unrepairable DNA damage may drive cells toward apoptosis or cellular senescence [107]. In this sense, in higher organisms, the DDR prevents neoplastic transformation, ensuring the termination of cellular proliferation and the removal of severely damaged cells [108].

Cells may display senescence-associated heterochromatin foci (SAHF), detectable with immunostaining techniques (Figure 3), which result from the association of the retinoblastoma (Rb) tumor suppressor and heterochromatin protein (HP) 1, DNA methyltransferase (DNMT) 1, or the suppressor of variegation 3–9 (Suv39) methyltransferase, which together form repressive complexes for the E2 transcription factor (E2F) 1 gene targets [109]. Moreover, the DNA damage caused by senescence inducers provokes the formation of persistent nuclear foci or DNA-SCARS characterized by chromatin alterations that reinforce cellular senescence [110]. In classical or normal reparative conditions, this process forms early foci that can be detected by γ-H2Ax or 53BP1 staining; in successful normal DNA repair, their expression rapidly disappears, while in senescence, these structures persist longer because of the elevated damage to the DNA, thus allowing the DNA-SCARS formation [111]. Moreover, DNA damage is sensed by ataxia telangiectasia-mutated (ATM), an essential response kinase coordinating checkpoint, and senescence responses. ATM is activated by either DNA breaks or oxidative stress and plays an essential role in the senescence response by phosphorylating and stabilizing p53 [112–116]. From a molecular viewpoint (Figure 3), the upregulations of the tumor suppressor Rb-$p16^{INK4A}$ and p53-$P21^{Cip1}$ pathways (Figure 3) are molecular hallmarks that participate in the induction of cellular senescence by downregulating cyclin/CDK and inhibiting E2F1 activity [62, 117]. In addition, downregulation of the nuclear lamina protein lamin B1 has also been postulated as a feature of the senescent phenotype [118, 119].

Even though cellular senescence implies a permanent cell cycle arrest, these cells remain metabolically active, earning the nickname "zombie" cells, and interact with other cells in the tumor microenvironment by cell-cell interaction or via the senescence-associated secretory phenotype (SASP), influencing the fate of neighboring cells via bystander effects (Figure 3) [120, 121]. The SASP encompasses a plethora of cytokines, growth factors, and proteases such as interleukin- (IL-) 1, IL-6, IL-8, growth-regulated oncogene (GRO) α/β, granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor binding proteins (IGFBPs), matrix metalloproteinases- (MMP-) 1, MMP-3, and MMP-10, intercellular adhesion molecule- (ICAM-) 1, and plasminogen activator inhibitor type 1 (PAI-1) [122, 123].

Nevertheless, a significant challenge is to typify senescence cells accurately. None of the above markers can be considered universal, and typifying senescence requires different phenotypical, biochemical, and molecular measurements. Recently, a combination of cytoplasmic markers, such as SA-β-gal, proliferation markers that are nuclear-localized, including $p16^{INK4AA}$, $p21^{WAF1/Cip1}$, Ki67, and SASP expression, have been recommended to standardize senescence characterization (Figure 3) [61].

Although CIS often is associated with tumor growth inhibition and regression [74], senescent cells may remain after the termination of onco-therapies and promote tumor progression by the SASP because they promote tumor cell dormancy, therapy resistance, and cancer relapse [64, 124–128]. In addition, SASPs influence the progression of surrounding nonsenescent tumor cells and metastasis by influencing the tumor microenvironment by factors that may promote the epithelial-to-mesenchymal transition (EMT), thus accelerating migration, invasion, and cancer cell malignancy features [129–132].

4. Cellular Oxidative Stress and Hydroxyurea

Reactive oxygen species (ROS) are constantly generated in normal physiological conditions, and they are eliminated by scavenging systems, thus maintaining cellular REDOX homeostasis. Meanwhile, dysbalance of this homeostasis due to aberrant ROS production or antioxidant decrease contributes to tumor progression and is a hallmark of several types of cancer (Table 1) [133, 134]. Moreover, exacerbated ROS levels result in biomacromolecular damage of proteins, lipids, and DNA among others, which promotes cellular

Table 1: Reactive oxygen species and hydroxyurea main functions and effects on tumorigenesis.

Function	Cellular and molecular effects	Ref.
Reactive oxygen species		
Intracellular signaling pathway regulation	Cell proliferation and survival, cell motility, invasiveness, and metastasis	[140]
Senescence induction	Telomere-dependent mechanism and telomere-independent mechanism (i) Double-strand DNA breaks induction (ii) DNA lesions due to 8-oxo-2′-deoxyguanosine generation (iii) Genomic instability (iv) Gene mutations implicated in the following: (a) Inhibition of tumor suppressor genes (b) Activation of oncogenes	[143–147]
Regulation of cellular proliferation	H_2O_2, superoxide ($O_2\cdot^-$), and hydroxyl radical (OH·) reduce cell proliferation	[148, 149]
Hydroxyurea and reactive oxygen species		
Cytotoxicity	Cytotoxicity and teratogenicity due to radical chain reactions, via H_2O_2, initiated by HU hydroxylamine group to form R-HN̈OH$^+$ radical and generation of NO	[150, 151]
DNA damage by increasing oxidative stress	Thymidine and cytosine damage via increasing NO and H_2O_2 and fork collapse	[6, 45, 55, 152]
Nitric oxide generation	RNR enzyme inhibition via NO and nitrosyl radical ·NO production	[45, 153, 154]
Scavenger protein inhibition	Downregulation of superoxide dismutase-2, peroxiredoxin-1, and Sirtuins	[154–156]

senescence and aging and is associated with the physiopathology of several age-associated diseases [135].

ROS comprise a family of highly reactive molecules that regulate normal cellular conditions by fine control of the generation/consuming rate. In contrast, in cancer, a dysregulated oxidative stress is produced that contributes to the chemical damage of proteins, lipids, and DNA and tumorigenesis promotion [136]. From a molecular viewpoint, ROS are small molecules derived from the oxygen comprising free radical and nonfree radical oxygen intermediates, ions, or molecules that have a single unpaired electron in their outermost shell of electrons. Moreover, ROS are constantly generated inside cells by enzyme complexes or as by-products of REDOX reactions, including those underlying mitochondrial respiration [137, 138]. These molecules include oxygen radicals, such as superoxide anion, hydroxyl, peroxyl, and alkoxyl, and nonradical molecules that are either oxidizing agents or easily converted into radicals, such as hypochlorous acid, ozone, singlet oxygen, and hydrogen peroxide. In addition, this oxygen-containing reactive species can combine with nitrogen to generate nitrogen-containing oxidants such as nitric oxide and peroxynitrite that belong to the family of reactive nitrogen species (RNS) [136, 138]. Furthermore, the REDOX dysbalance in cancer cells is generated by increased cellular metabolic activity, mitochondrial dysfunction, deregulated cellular receptor signaling, peroxisome activity, oncogene activation, cyclooxygenase lipoxygenases, and thymidine phosphorylase. In addition, the contribution to the REDOX dysbalance of these factors may depend on the malignant stage of the cancer cells and their interaction with tumor stroma and infiltrating immune cells [139, 140]. Furthermore, cellular superoxide anions form mainly because of the NADPH oxidase (NOX) family [141]. Five forms of NOXs have been found: the small GTPase Rac1-dependent NOX1, NOX2, and NOX3, and the small GTPase Rac1-independent NOX4 and NOX5 [142].

ROS participate in different aspects of tumor development and progression; they regulate intracellular signaling pathways involved in cell proliferation and survival while also influencing cell motility, invasiveness, and metastasis and regulating inflammatory responses within the tumor stroma and in angiogenesis [140]. Furthermore, ROS contribute to determining mammalian cells' senescent cellular fate [143, 144]. These oxygen-containing reactive species can promote cellular senescence by telomere-dependent mechanisms and telomere-independent mechanisms involving unrepairable single or double-strand DNA breaks [145, 146]. Moreover, their excessive levels generate DNA lesions by forming 8-oxo-2′-deoxyguanosine, which accumulates in senescent human cell cultures and aging mice. Consequently, this DNA damage generates genomic instability, DNA mutations, and tumor development [147]. Therefore, ROS produce genomic alterations such as point mutations and deletions, which may inhibit tumor-suppressor genes while activating and inducing the expression of oncogenes to further contribute to the enhancement of cancer cell malignancy [143].

On the other hand, ROS also regulates cellular proliferation, which depends on their levels and duration of exposure. In this sense, most cytostatic/cytotoxic anticancer drugs inhibit cancer cell proliferation and cell survival by promoting ROS generation [148, 149]. For instance, both H_2O_2 and its dismutation product superoxide ($O_2\cdot^-$) reduce cancer cell proliferation, while H_2O_2 may also form, via Fenton reaction, the hydroxyl radical (OH·) that highly inhibits cell proliferation [149].

Although HU can enhance cellular oxidative stress, the intimate molecular mechanism is not well understood. Some earlier studies have suggested that this drug may exert cytotoxic effects through radical chain reactions via H_2O_2 and initiated by its hydroxylamine group. Conversely, radical scavengers substantially reduce the cytotoxic and teratogenic HU activities [150, 151]. Moreover, HU causes DNA damage to thymidine and cytosine residues via increasing H_2O_2, in part by inducing ROS via provoking a fork collapse. Moreover, this agent induces mutagenic DNA lesions in V79 Chinese hamster cells, likely due to the generation of H_2O_2 [6, 45, 57, 152].

Moreover, nitric oxide radical (·NO), generated upon the 3-electron oxidation of the drug, may be responsible for many of its pharmacologic effects, including the RNR enzyme inhibition [153, 154]. Nevertheless, recent analyses indicated that HU might downregulate the expression of scavenger proteins, such as superoxide dismutase (SOD) 2 and peroxiredoxin-1 (PRDX1), and regulatory oxidative stress proteins, such as Sirtuin- (Sirt-) 3 (Table 1) [154–156]. Although the involved molecular mechanisms by which HU regulates the expression of these proteins have not been well elucidated so far, the induced deficiency of these oxidative stress regulatory proteins significantly contributes to the elevation of ROS by HU and the establishment of cellular senescence.

5. Hydroxyurea and Cellular Senescence

HU inhibits proliferation in several organisms and cell lines. At therapeutically relevant levels, HU mainly induces cell proliferation arrest in the S cell cycle phase because of the decrease in dNTPs by RNR enzymatic activity inhibition [157, 158]; this causes a reduction of DNA polymerase movement at replication forks that generate a DNA replication stress [6, 102]. In cancer therapy, this agent is frequently used as an antitumor agent because of its cytoreduction functions. Moreover, HU belongs to the family of antimetabolite drugs that can induce premature cellular senescence from interfering with the crucial synthesis pathways required for DNA duplication (Figure 4) [102, 128].

One of the first observations that HU may promote senescence-like phenotype in cancer cells was made in the human erythroleukemia K562 cell line. K562 cells underwent cell proliferation arrest and positivity to SA-β-gal activity after seven days of HU treatment. Moreover, the treatment increased the expression of the cyclin-dependent kinase inhibitors $p16^{INK4A}$ and $p21^{Cip1}$ [159]. Interestingly, since K562 cells are p53-deficient [160], HU-induced senescence can occur independently of p53 activity in these cells. Additionally, this agent also induces cellular senescence in rat hepatoma McA-RH7777 cells; after treatment, cells exhibited enlarged size, increased SA-β-gal positive staining, and a substantial reduction in cell proliferation as cells were arrested in the G_0/G_1 cell cycle phase. In this case, a substantial reduction in the cellular frequency at the G_2/M phase was observed. Cells undergoing HU treatment consistently expressed elevated levels of $p21^{Cip1}$ associated with cell cycle arrest at the G_1/S interphase [161]. Likewise, the drug promotes cellular senescence in neuroblastoma cell lines after a relatively long period of treatment, in part because of HU concentrations below 200 μM. After five weeks of treatment, more than 50% of the cells stained positive for SA-β-gal, and in this period, cells exhibited a reduction of telomere length that was 50% of the cells after ten weeks [162]. Although this pharmaceutical compound induces neuroblastoma cell senescence in vitro, it does not promote cell secretion of unfavorable SASPs, such as MMP-9, the monocyte-chemotactic protein- (MCP-) 3, the regulated-on activation normal T cell expressed and secreted (RANTES), and the vascular endothelial growth factor (VEGF). In contrast, it induces secretion of IL-6 and platelet-derived growth factor- (PDGF-) AA, involved in immuno-regulation and angiogenesis [80, 163–165].

Besides cancer cells, HU may affect nontransformed cells. For instance, in a model of foreskin fibroblast cells, treatment with the drug in the range of 400–800 μM provoked a reduction of cell proliferation and morphological changes similar to the findings in replicative cellular senescence; moreover, these changes were not reversible by removing the drug treatment. HU treatment induces SA-β-gal activity and p53 and $p21^{Cip1}$ expression along with Jun N-terminal kinase (JNK) activation. Moreover, because of HU treatment, senescence fibroblasts are protected from UV light-induced apoptosis [166]. Similar results were reported in a human embryonic fibroblast cell line; the treatment with this medical agent induced SA-β-gal and $p21^{Cip1}$; moreover, the elevated $p21^{Cip1}$ expression seemed due to increased protein stability rather than de novo synthesis. In addition, increased $p21^{Cip1}$ was independent of increased p53; thus, suggesting that in these cells, p53 activity was not implicated [167], which is concordant with the theory that p53 mainly transcriptionally activates $p21^{Cip1}$ expression [168]. In addition, the HU-induced senescence in mouse fibroblasts, determined by SA-β-gal activity, is increased by transcription factor c-Jun depletion, while c-Jun overexpression inhibits the senescence induced by the treatment and drives cells to cell death.

Meanwhile, the transcription factor JunB enhances HU-induced senescence by upregulation of their direct target $p16^{INK4A}$. These results suggest that the balance between the c-Jun and JunB transcription factors may determine the cellular response to the chemotherapeutic HU agent [169]. In addition, the chronic exposure of rat and human fibroblasts to low concentrations of the chemotherapeutic agent induced cellular senescence by a p53-dependent $p21^{Cip1}$ expression and increased SA-β-al activity, but independent of $p16^{INK4A}$. Moreover, HU induces reversible γH2A.X foci, indicating that replicational stress induced by HU promotes DNA strand breaks [58].

HU treatment also can induce postnatal subventricle neural stem cells (NSCs) to undergo cellular senescence [154]. In this case, elevated concentrations of the drug (at mM levels) cause persistent DNA damage evidenced by γH2AX foci formation and a consistently increasing number of SA-β-gal positive cells, as well as increased $p16^{INK4A}$, $p21^{Cip1}$, and p53 expression. Moreover, under HU treatment, cells suffered a reduction of proliferation as a

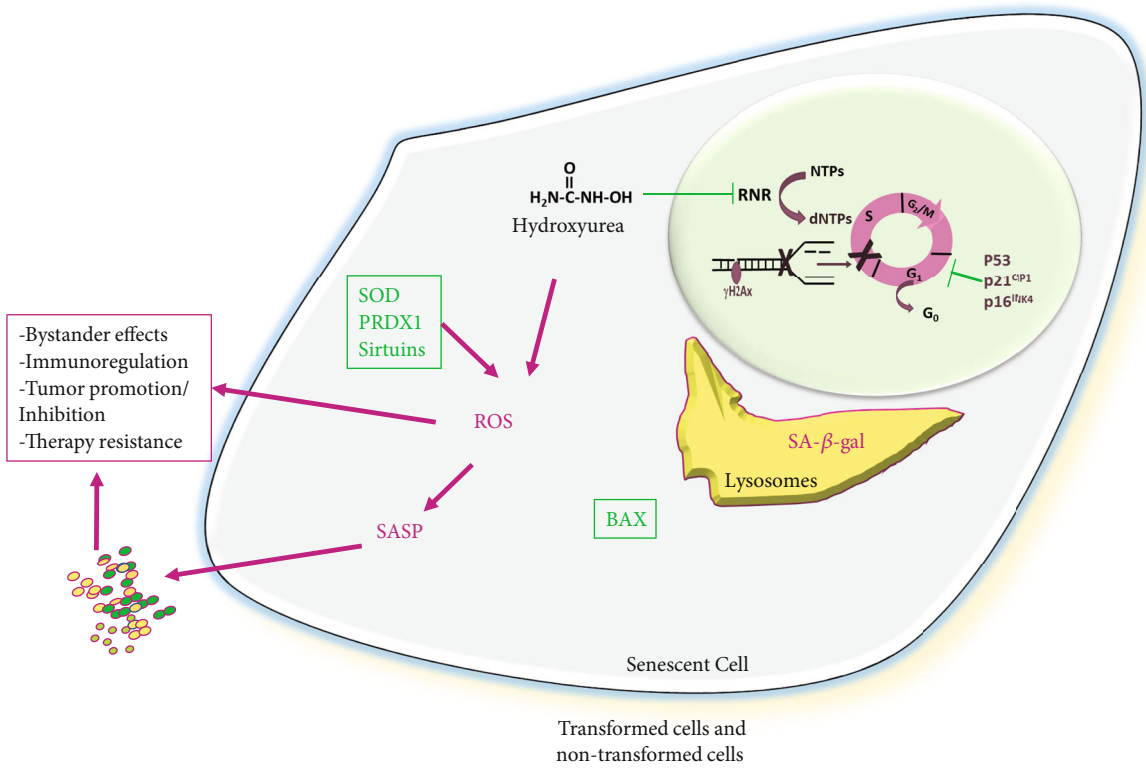

Figure 4: Overview of the main features of hydroxyurea-induced cellular senescence. Hydroxyurea, by inhibition of ribonucleotide reductase (RNR), dramatically reduces the synthesis of deoxyribonucleotides (dNTPs) from ribonucleotide substrates (NTPs). This dNTP pool reduction provokes a termination of DNA replication and may result in replication fork collapse. Furthermore, because of genotoxic HU action, DNA damage is generated, and phosphorylated histone H2AX (γH2AX) binding to DNA breaks is promoted. Cells may suffer an arrest at the S cell cycle phase, concomitant with increased expression of cell cycle inhibitors $p16^{INK4A}$, $p21^{Cip1}$, and p53, reinforcing the cell cycle inhibition. During senescence induction, cell size is enlarged, and lysosomal biogenesis is increased, as indicated by elevated levels of expression and senescence-associated-β-galactosidase (SA-β-gal). Along with DNA replication inhibition, augmentation of oxidative stress occurs as reactive oxygen species (ROS) expression levels are elevated, consistently reducing antioxidative stress protein superoxide dismutase (SOD) 2, peroxiredoxin (PRDX) 1, and Sirtuins that contribute to maintaining increased oxidative stress. Moreover, HU-induced senescent cells are refractory to apoptosis, in part from reduced expression of the proapoptotic BAX protein. Senescent cells are metabolically active, and they express and release a set of factors as part of the senescence-associated secretory phenotype (SASP). The SASP may profoundly influence surrounding cells and tissues through increased local and systemic inflammation and regulation of immune response, depending on SASP pattern, positively or negatively affecting tumor growth, and may also contribute to therapy resistance. Magenta words mean increased expression. Magenta arrows mean induction. Gree T-shape symbols mean inhibition. Green words mean reduced expression.

consequence of a cell cycle arrest at G_0/G_1. Furthermore, the treatment increased intracellular ROS levels along with a significant decrease in SOD2 and PRDX1. SOD2 is a main antioxidant enzyme that scavenges ROS in the inner mitochondrial matrix and acts as the first defense against mitochondrial oxidative stress [170], while PRDX1 is a thiol-specific peroxidase that scavenges hydrogen peroxide [171]. In addition, this pharmaceutical agent provokes a downregulation of Bcl-2-associated X protein (BAX), a critical proapoptotic factor that may contribute to the decreased apoptosis observed in senescent NSCs [154, 172]. In addition, HU-induced NSC cellular senescence is counteracted by α-glycerylphosphorylethanolamine (GPE) [173], which is a precursor biomolecule of phospholipid synthesis and exerts neuroprotective effects in human hippocampal cells [174]. For instance, GPE protects NSCs from the induction of DNA damage caused by phosphorylated γH2AX levels and rescues cell proliferation from HU inhibition. Furthermore, GPE highly reduces HU-induced SA-β-gal expression and activity and p53 and $p21^{Cip1}$ mRNA expression. Moreover, this chemotherapeutic agent increases the ADP/ATP ratio that indicates mitochondrial energy metabolism impairment, while GPE restores the physiological ADP/ATP ratio and significantly reduces HU-induced ROS levels. GPE also consistently inhibits the ROS-responsive NF-κB signaling [175]. Thus, GPE protects NSCs from HU-induced cellular senescence, indicating that it might function as an antiaging compound for NSCs [173].

HU can also induce cellular senescence of mesenchymal stem/stromal cells (MSCs). MSCs are multipotent cells characterized by their ability to differentiate into adipocytes, chondrocytes, and osteoblasts; their expression of surface markers CD73, CD90, and CD105; and their lack of hematopoietic lineage markers [176, 177]. They are also present in the tumor microenvironment, where they support the growth of tumor cells, activate mitogen and stress signaling,

and increase resistance to cytotoxins [178, 179]. HU at relatively high levels inhibits dental follicle-derived MSC proliferation and clone formation capacity along with increased DNA double-strand breaks indicated by γH2AX foci formation; additionally, it induces SA-β-gal activity and a higher expression level of p53, $p21^{Cip1}$, and $p16^{INK4A}$. These effects are accompanied by reducing MSC differentiation toward adipogenic, chondrogenic, and osteogenic lineages.

Moreover, senescence induction by HU increases ROS levels along with the downregulation of SOD2 [155]. Similarly, peripheral blood MSCs (PB-MSCs) are also targeted by this agent [180]. HU induces a senescence-like phenotype in PB-MSC as it provokes substantial cell morphology changes accompanied by SA-β-gal and $p16^{INK4A}$ expression with a discrete effect on $p21^{Cip1}$ expression. The treatment with the drug at therapeutically relevant concentrations (200 μM) strongly induces cell cycle arrest to the S cell cycle phase; consistent with that, in the presence of HU, cells progress from G_1 to the S phase at a normal rate and are arrested in the early S phase [58]. This pharmaceutical compound also increases intracellular ROS levels that contribute to senescence induction because oxidative stress scavengers, N-acetylcysteine, and NOX inhibitor apocynin inhibit cellular senescence and partially protect PB-MSC proliferation from inhibition by HU. Furthermore, HU-induced senescent PB-MSCs significantly inhibit the proliferation of erythroleukemia cells by secreting TGF-β1 and elevated ROS production. Thus, senescent PB-MSCs may shift from a tumor-promoter activity to a tumor-suppressive function [180].

As stated, HU during senescence induction promotes an elevation of cellular ROS in part because of downregulation of SOD2, and recently, it was reported that this drug could also inhibit the expression of Sirt-3 (Figure 4) [156]. Sirt-3 is a mitochondrial deacetylase that regulates major mitochondrial biological processes, including ATP generation, ROS detoxification, nutrient oxidation, mitochondrial dynamics, and the unfolded protein response [181, 182]. Sirt-3 also deacetylates and thereby activates SOD-2 [183]. HU induces mouse embryonic fibroblast (MEF) senescence and increases ROS levels and Sirt-3 and SOD2 downregulation. Interestingly, adjudin is a compound derived from the anticancer drug lonidamine that acts through Sirt-3 activation [184]. Adjudin delays HU-induced cellular senescence reducing ROS levels by Sirt-3 upregulation [156]. Although it reduces the anti-ROS proteins Sirt-3 and SOD-2 expression during cell senescence induction, no molecular mechanism implicated in their downregulation has yet been elucidated. Nevertheless, it is important to reveal the underlying mechanistic pathways of elevated ROS levels due to HU treatment. Moreover, adjudin, due to its antisenescence function, may contribute to the therapy for age-associated diseases and CIS.

Similarly, 1,5-isoquinolinediol (IQD), a poly (ADP-ribose) polymerase (PARP1) inhibitor, protects MEF cells from HU-induced senescence [185]. PARPs perform poly(-ADP-ribosyl)ation of proteins as an immediate cellular response to genotoxic insults induced by ionizing radiation, alkylating agents, and oxidative stress [186]. HU accelerates the MEF replicative senescence rate by inducing oxidative stress paralleled to increasing PARP1 and lamin A expression, while IQD effectively suppresses the senescence rate by decreasing the activity of PARP1 [185]. Noticeably, the increased expression and activity of PARP1 rapidly consume the NAD+ necessary for Sirt-1 function, so the decreased Sirt-1 activity results in increased oxidative stress. Thus, pharmacological PARP1 inhibition may restore NAD+ levels and Sirt-1 activity and normalize oxidative metabolism [187], which may help control the prosenescence function of HU and prevent chemotherapy-associated accelerated aging in cancer survivors [188].

6. Concluding Remarks

HU as a nonalkylating antiproliferative agent is still used to manage a variety of disease conditions in both neoplastic and nonneoplastic settings, and it is listed as an essential medicine by WHO. This drug can function as a cytoreductive agent because of its cytostatic properties; in this sense, as is analyzed in this review, HU can induce cellular senescence in both cancer cells and nontransformed cells, which profoundly affects tumor growth and homeostatic function of normal cells. Mechanistically, this compound functions as an antimetabolite agent by acting on RNR and affecting the generation of the dNTP pools necessary for DNA synthesis and duplication. The dNTP deficiency may cause fork collapse associated with DNA damage and ROS generation, which contributes to establishing a cellular senescence phenotype. What is the molecular mechanism by which HU increases ROS? It is a relevant question to address experimentally; cells under treatment may exhibit reduced expression of antioxidative stress, SOD2, PRDX1, and Sirtuins that contribute to the enhancement and stabilization of elevated ROS levels. For instance, repression of SOD2 may occur at the level of epigenetic regulation [189], and HU may promote epigenetic modifications along with regulation of several intracellular signal transductions, such as MAPK, PKG, and PKA signaling [190], which, in part, may explain the reduced expression of SOD2 during the increase in ROS levels and the cellular senescence due to HU treatment.

Different strategies have emerged to eliminate CIS cells because of the need to eliminate tumor cells and nontransformed dysfunctional cells. To this end, senolytic strategies have been developed to target CIS-transformed cells and, potentially, the nontransformed senescent cells without affecting normal proliferating cells [191]. In addition, the increased ROS levels that contribute to HU-induced cellular senescence are valuable targets for developing therapeutic strategies to improve the cytotoxic function of the drug, which may shift cells from the senescence response toward cell death fate [192]. Understanding the delicate balance between cellular senescence and the beneficial anticancer function of HU is vital to improving the current therapies to impact the life quality of patients and control the undesirable premature aging caused by chemotherapy.

Data Availability

No datasets were generated or analyzed during the current study.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Research Article

Increased ROS-Dependent Fission of Mitochondria Causes Abnormal Morphology of the Cell Powerhouses in a Murine Model of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in humans and remains to have a fatal prognosis. Recent studies in animal models and human ALS patients indicate that increased reactive oxygen species (ROS) play an important role in the pathogenesis. Considering previous studies revealing the influence of ROS on mitochondrial physiology, our attention was focused on mitochondria in the murine ALS model, wobbler mouse. The aim of this study was to investigate morphological differences between wild-type and wobbler mitochondria with aid of superresolution structured illumination fluorescence microscopy, TEM, and TEM tomography. To get an insight into mitochondrial dynamics, expression studies of corresponding proteins were performed. Here, we found significantly smaller and degenerated mitochondria in wobbler motor neurons at a stable stage of the disease. Our data suggest a ROS-regulated, Ox-CaMKII-dependent Drp1 activation leading to disrupted fission-fusion balance, resulting in fragmented mitochondria. These changes are associated with numerous impairments, resulting in an overall self-reinforcing decline of motor neurons. In summary, our study provides common pathomechanisms with other ALS models and human ALS cases confirming mitochondria and related dysfunctions as a therapeutic target for the treatment of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common systemic disease of the motor system. The annual incidence rate in Europe is 2.2 per 100,000 inhabitants per year [1]. It is mainly classified in a familial and sporadic form. The sporadic form of ALS (sALS) is clearly the most frequent with a share of about 90-95%, followed by the familial form (fALS) with about 5-10%. The disease is characterized by bilateral degeneration of cells from *tractus corticospinalis*, in the nuclei of cranial motoric nerves as well as motor neuronal cells in the anterior horn of the spinal cord [2]. The degeneration of the first motor neuron leads to progressive spastic paresis and painful muscle cramps combined with hyperreflexia, pseudobulbar paralysis, and pyramidal path signs [3]. In the course of time, these symptoms are masked by the degeneration of the second motor neuron. The main symptoms are muscular atrophy and weakness, fasciculation (especially of the tongue), and progressive respiratory insufficiency [4]. Ultimately, respiratory failure leads to death within 2 to 5 years after diagnosis in most cases. Although the disease was already described and diagnosed in 1869, no curative therapy has been developed to date [5]. Supportive medications like Riluzole, which has antiglutamatergic

effects, and Edavarone, which is supposed to reduce oxidative stress, can only prolong survival by a few months [6].

Only a few genes have been associated with ALS as causative factors so far. The most frequently altered genes in ALS patients are *C9orf72*, *SOD1*, *TARDBP*, and *FUS* [7, 8]. And new mutations are constantly being linked to ALS, such as mutations in profilin 1 (*PFN1*) [9], specific kinesin isoforms (*KIF5A*) [10], or the chaperon Sigma-1 receptor (*SIGMAR1*) [11]. However, the genetic origin of most ALS cases remains unclear [7]. In the sporadic form, even more than 80% of ALS cases remain without an uncovered genetic cause. However, current knowledge suggests that ALS is caused by a complex interplay of different pathomechanisms, including the motor neurons themselves and interactions with neighboring cells such as microglia and astrocytes [12]. At a cellular level, protein misfolding and aggregation, glutamate-induced excitotoxicity, neuroinflammation, and deficient axonal transport are among the known pathological events in ALS [13]. In recent years, mitochondrial dysfunction and oxidative stress have increasingly become the focus of science attention and are now considered to play a key role in the pathogenesis cascade of ALS [14].

In several models used to study ALS pathology, proteins of the antioxidative system and oxidative stress play important roles. An example for this is the murine ALS model of the superoxide dismutase 1 (SOD1) [15]. About 2.5-23% of fALS and 0.44-7% of sALS cases are related to this *SOD1* gene, which encodes for a cytosolic antioxidative enzyme [16]. Further studies in tissues from ALS patients and animal models have demonstrated dysfunctional mitochondria with consequently increased oxidative stress, as reviewed in Carrì et al.'s study [17]. This is not only present on functional but also on morphological level. Based on *postmortem* studies from ALS patients and transgenic $SOD1^{G93A}$ mice, fragmentation of the mitochondrial network is described on a morphological level [18, 19]. Deregulated fission and fusion-related enzymes like mitochondrial Dynamin-like 120 kDa protein (Opa1), Mitofusin1 (Mfn1), mitochondrial fission 1 protein (Fis1), and Dynamin-1-like protein (Drp1) were detected as reasons for the fragmentation in $SOD1^{G93A}$ transgenic mice [20]. This underlines the importance of the fusion/fission balance and oxidative stress in the pathogenesis of ALS.

For our investigations, we used the wobbler mouse as an ALS model. The wobbler mouse was first described by Falconer in 1956 and arose from the C57BL/Fa mouse strain by a spontaneous mutation. Later, a loss-of-function mutation in the *VPS54* gene was identified as the genetic cause of the wobbler ALS disease [21]. Recently, several mutations of *VPS54* have been discovered in human ALS patients with Project MinE (http://databrowser.projectmine.com/, Accessed 11 May 2021) indicating an involvement of this gene in the pathogenesis of some ALS cases. Nearly all phenotypic and cellular symptoms of ALS, like motor defects, tremor, muscle weakness and atrophy, degeneration of the 1st and 2nd motor neuron, astrogliosis, defects in vesicle transfer, and axonal transport, are present in homozygous wobbler mice [22–24]. Wobbler disease develops within three typical stages—presymptomatic (p0-p19), evolutionary (p20-p39), and stable (>p40) stage. In the presymptomatic stage, both genotypes show no clinically visible differences. In the evolutionary stage, typical symptoms such as head tremor, motor defects, and muscle weakness develop rapidly. At a cellular level, degeneration of the upper and lower motor neurons, reduced axonal transport, and mitochondrial dysfunction can be observed [24, 25]. In the stable stage starting from p40, the symptoms stagnate [26]. Mitochondrial dysfunctions were detected at various time points during wobbler disease. In detail, a restricted function of complexes I, III, and IV of the mitochondrial electron transport chain and a reduced oxygen consumption rate of complex I were observed on isolated mitochondria of wobbler brain [27]. Dave et al. [28] confirmed these differences and demonstrated their presence even at earlier time points of the disease. In accordance with this, Santoro et al. [29] demonstrated a reduced oxygen consumption rate of complexes I and IV of the mitochondrial electron transport chain as well as a reduced activity of complex I in isolated mitochondria of the cervical part of the spinal cord. Furthermore, decreased activities of respiratory complexes I, II, and III were found in mitochondria of the cervical and partially (complex I only) lumbar spinal cord [30]. Previous studies in $SOD1^{G93A}$ transgenic mice found that oxidative stress leads to fragmentation of the mitochondrial network and ultimately neurodegeneration [19]. This fragmentation was caused by a deregulation of fission and fusion-related enzymes due to increased oxidative stress [20]. Our previous study revealed that increased levels of reactive oxygen species (ROS) are present in the cervical spinal cord of wobbler mice [31]. Here, we aimed to decipher the consequences of increased ROS on mitochondrial network, individual mitochondrial parameters, and mechanisms of mitochondrial dynamics in motor neurons of wobbler mice in order to reveal common parallel pathomechanisms between different ALS models. These findings may open new treatment strategies which are independent of the present genotype and thus beneficial to a larger ALS patient cohort.

2. Materials and Methods

2.1. Animals. All procedures were conducted under established standards of the German federal state of North Rhine Westphalia, in accordance with the European Communities Council Directive 2010/63/EU on the protection of animals used for scientific purposes. Animal experiments were conducted according to the German animal welfare regulations and approved by the local authorities (registration number Az. 84-02.04.2017.A085). The used mouse strain is C57BL/Fa carrying the wobbler point mutation in the *VPS54* gene. Breeding and genotyping were carried out as described previously [22]. The mice were kept in a 12 h night/day cycle and had access to food and water *ad libitum*. Cervical spinal cord tissues from WT and WR animals were collected at the age of p20 and p40 and used for further experiments. All experiments were exclusively carried out with 3-10 homozygous WT or WR mice. Both genders were used. Heterozygous animals were used for breeding.

2.2. Motor Neuron Enriched Dissociated Cell Culture of the Ventral Horn. The protocol for the cultivation of dissociated

spinal cord cell cultures was performed as described before [26]. In brief, homozygous mice were decapitated at the age of p40 and spinal cords were removed. After removing meninges and separating the anterior horns, tissues were cut into small fragments and digested with 36 U/ml papain (#LS003119, Cell Systems, Germany) isolation medium [0.5 mM GlutaMax (#35050061, Thermo Fisher Scientific, Germany), 100 U/ml Penicillin/Streptomycin (#P4333, Merck, Germany), and 2% B27 supplement (#17504044, Thermo Fisher Scientific, Germany) in Hibernate A (#A1247501, Thermo Fisher Scientific, Germany)] for 10 min at 37°C. After trituration of the tissue, the isolated cells were separated by density gradient centrifugation on an OptiPrep (#1114542, Progen, Germany) density gradient according to Zwilling et al. [26]. The cells from fractions 2 and 3 were pelletized by centrifugation (244 g for 6 min at 10°C) and plated in motor neuron feeding medium [30% C2C12 myocyte-conditioned medium [32], 0.5 mM glutamine (#G7513, Merck, Germany), 100 U/ml Penicillin/Streptomycin, 2% B27 supplement, 125 mM cAMP (#A6885, Merck, Germany), 1 ng/ml BDNF (#CYT-207, Prospec, Israel), and 0.1 ng/ml GDNF (#CYT-305, Prospec, Israel) in Neurobasal A (#10888022, Thermo Fisher Scientific, Germany)] at a density of 70.000 cells per well onto poly-D-lysine- (50 μg/ml, #P7280, Merck, Germany) coated high precision glass cover slides (12 mm). The cells were cultivated in vitro for 10 days at 37°C and 5% CO_2 with a medium change after 2 to 3 days.

2.3. Immunofluorescence Staining. Immunofluorescence staining have been performed in dissociated motor neuronal enriched cultures. After 10 days *in vitro*, cells were incubated in neuron feeding medium containing CellTracker (10 μM, #C2925, Thermo Fisher Scientific, Germany) and MitoTracker (100 nM, #M22426, Thermo Fisher Scientific, Germany) for 40 min under normal incubation conditions. Subsequently, the cells were fixed with 4% paraformaldehyde, and nuclei were stained with DAPI (#D9542, Merck, Germany). To investigate the mitochondrial network of motor neurons from WT and WR, cultures were imaged with a superresolution microscope with structured illumination (Zeiss Elyra PS.1 LSM880, Carl Zeiss Microscopy GmbH, Germany) in combination with a 63x oil immersion objective (Plan-Apochromat 63x/1.4 Oil DIC, Carl Zeiss Microscopy GmbH, Germany) equipped with respective filter sets. We have focused here on mitochondria in the perinuclear and soma region as these contain the largest number of mitochondria and are most critical in terms of mitochondrial degradation, making them best suited for a description of the network. Imaris 9.2.1 (Oxford Instruments, UK) surface and spot function were used for the evaluation of parameters describing the mitochondrial network precisely. First, the motor neuron was manually marked, and a region of interest was defined. Outside this region of interest, all voxels were set to 0. This guaranteed that results were not influenced by cells in the surrounding area. The spot function forms spheres around the individual signals, while the surface function maps the structure of the mitochondrial chains. For the surface function, we set a threshold value of 150 and for the spot function a threshold value of 200 and the quality filter type. At least 50 motor neuronal cells were measured for each genotype. All obtained parameters were quantitatively evaluated using GraphPad Prism 7 software (GraphPad Software, USA). Data are presented as the mean values ± SEM. The Kolmogorov-Smirnov normality test was used to confirm normal distribution. Student's t -test was performed for significance testing between the two genotype groups, and values with $p < 0.05$ were considered to be significant.

2.4. Transmission Electron Microscopy. After describing the mitochondrial network in the soma of motor neurons with aid of immunofluorescence staining, the question arose if the network structure is reflected in abnormal individual mitochondria. To close this gap, TEM studies were performed. The embedding protocol was based on Krause et al.'s study [33]. Mice were anaesthetized with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) and transcardially perfused with 2.5% glutaraldehyde (#G5882, Merck, Germany) in phosphate buffer (PB). After incubation of the tissue in Dalton solution [1 g OsO4 (#19134, Electron Microscopy Sciences, Belgium) solved in 100 ml 5% potassium dichromate solution (#7953, Roth, Germany)] for 2 h, tissue was washed with PB. Next, specimens were dehydrated through an ascending ethanol series, starting with 50%-ethanol, followed by incubation in 70% ethanol, 1% uranyl acetate (#21447, Polyscience Inc., England) and 1% phosphotungstic acid (#455970, Merck, Germany) solution overnight at 4°C. The next day, dehydration continued with an ascending ethanol series (80-100%). The specimens were carefully transferred into epoxy resin. This was accomplished by first incubating the tissue in propylene oxide (#807027, Merck, Germany), followed by an ascending series of propylene oxide and EPON mixtures. This embedding procedure started with propylene oxide/EPON in a 3 : 1 ratio, followed by a 1 : 1 ratio, and ended with a 1 : 3 ratio. Finally, specimens were penetrated by pure EPON overnight at 20°C. On the third day of embedding, EPON was renewed. After all, EPON embedded specimens were allowed to polymerize at 60°C for two days. EPON consists of glycidether (#21045.02, Serva, Germany), methylnadic anhydride (#29452.02, Serva, Germany), 2-dodecenylsuccinic acid anhydride (#20755.01, Serva, Germany), and 2,4,6-tris(dimethylaminomethyl)phenol (#36975.01, Serva, Germany) in a 5.4 : 3.8 : 1.84 : 1 mixture. Ultrathin slices (70 nm) were cut with an Ultracut E Reichert-Jung (Leica Microsystems GmbH, Germany) with a DiATOME histo diamond knife (45°, 6 mm, MX559; Diatome AG, Switzerland). Philipps EM 420 (Philips, Netherlands) and ImageJ 1.51 s (National Institutes of Health, USA) were used for the evaluation of single mitochondria in detail as described below.

2.5. Analyses of Morphological Parameters. To describe the morphology of mitochondria in the soma of motor neurons, ultrathin sections (70 nm) of the cervical spinal cord were prepared, and the area of the anterior horn was magnified. Overview photographs were taken to identify the motor neurons. Therefore, motor neuron-specific characteristics such as size, shape, nucleus, soma texture, and Nissl bodies were

considered. Next, the number of mitochondria within a motoneuronal soma was counted. In the next step, the area around the nucleus was highly magnified and each mitochondrion within the motor neuron was captured. In order to describe the mitochondrial morphology in detail, various parameters were evaluated which have turned out to be proven for describing mitochondria in a previous study [34]. In detail, the following parameters were measured:

(i) Surface area (in nm^2)

(ii) Perimeter in 2D (in nm)

(iii) Aspect ratio (major to minor axis)

(iv) Feret's diameter (longest distance in one single mitochondrion)

(v) Roundness (rated by $4 * \text{area}/\pi * \text{major axis}^2$)

(vi) Circularity (rated by $4\pi * \text{area/perimeter}^2$)

(vii) Circularity value of 100% expressing a perfect circle

(viii) Elongated mitochondria having a circularity value closer to 0

Obtained parameters were quantitatively evaluated using GraphPad Prism 7 software (GraphPad Software, USA). The data are presented as mean values ± SEM. The Kolmogorov-Smirnov normality test was used to confirm normal distribution. Student's *t*-test was performed for significance testing between the two genotype groups, and values with $p < 0.05$ were considered to be significant.

2.6. TEM Tomography. During the evaluation of TEM images, the assumption arose that the IMM and crista structure of wobbler mitochondria seems to be altered. To examine these changes in detail, we studied the mitochondria with TEM tomography. First, mice were anaesthetized and transcardially perfused with 2% formaldehyde (#15714-S, Electron Microscopy Sciences, USA), 2.5% glutaraldehyde (#G5882, Merck, Germany), and 2 mM $CaCl_2$ in 0.15 mM cacodylate buffer. In the subsequent step, samples were stained with 2% osmium tetroxide and 1.5% potassium ferrocyanide in 0.15 mM cacodylate buffer for 1 h. Further, specimens were treated with 1% thiocarbonohydrazide (#T2137, Merck, Germany) for 25 min, 2% osmium tetroxide (#0972B-6, Polyscience Inc., England) for 30 min, and finally 2% uranyl acetate (#21447, Polyscience Inc., England) overnight at 4°C (each solved in H_2O). The next day, the samples were stained with 0.66% lead nitrate (#HN32.1, Carl Roth, Germany) in 3 mM aspartic acid (#A9256, Merck, Germany) for 30 min. Next, samples were dehydrated by incubation in an ascending ethanol series starting with 30% ethanol, followed by 50%, 70%, 80%, and 96%, and finally pure ethanol. The samples were then briefly immersed in propylene oxide (#807027, Merck, Germany) and afterwards in a Durcupan (#44610, Merck, Germany)/-propylene oxide mixture in a 1 : 2 and subsequent 3 : 1 ratio. Finally, the samples were embedded overnight in pure Durcupan and then polymerized in fresh Durcupan at 60°C for 3 days. Slices (thickness approximately 200 nm) were cut with an Ultracut E Reichert-Jung (Leica Microsystems GmbH, Germany) with a DiATOME histo diamond knife (45°, 6 mm, MX559; Diatome AG, Switzerland). Single axis tilt series were recorded on a JEOL JEM-1400 Plus transmission electron microscope (JEOL, Japan) operating at a 120 kV with a LaB6 filament and equipped with a 4096×4096 -pixel CMOS camera (TemCam-F416, TVIPS, Germany). Automated image acquisition (16 Bit resolution, 4096×4096 pixels, pixel size: 1,213 nm) over an angular range from -60° to 60° with 1° step size was performed using the software serialEM 3.58 (University of Colorado, USA) [35].

2.7. 3D Reconstruction of Mitochondria. Image alignment and reconstruction by filtered back projection was carried out using the software package IMOD 4.9.7 [36]. A 3D model was generated by manual segmentation of the reconstructed image stack with the segmentation feature of 3dMod from the IMOD package. On each image plane, individual objects with corresponding contours specific to the mitochondrial structures were assigned and exported as a surface mesh. Small mismatches and failures in the mesh were corrected with the software MeshLab 2020.07 (Institute of Information Science and Technology, Italy), and the final result was rendered by a raytracing algorithm implemented in the software Blender 2.83.2 (Blender Foundation, Netherlands).

2.8. qPCR. Since our previous studies revealed an altered mitochondrial network with smaller individual mitochondria, we intended to take a closer look at the fusion- and fission-related mechanisms. To accomplish this, qPCR and Western blotting were performed. Total RNA (tRNA) was extracted from the cervical spinal cord tissue of WT and WR mice at p40 using NucleoSpin miRNA Kit (#740971, Macherey-Nagel, Germany) according to the manufacturer's protocol. cDNA synthesis was performed with a reverse transcription system (#A3500, Promega, USA). Following the manufacturer's protocol, 1 μg tRNA and oligo(dT)15 primer were used. The cDNA was stored at -20°C until use. Standardized quantitative real-time PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). GoTag qPCR Master Mix (#A6001, Promega, USA) was used with 50 ng cDNA and the corresponding primer sets (0.7 μM each). The following primer sequences were used: *GAPDH*—5′-GGA GAA ACC TGC CAA GTA TGA-3′ (sense) and 5′-TCC TCA GTG TAG CCC AAG A-3′ (antisense), *Mfn1*—5′-AGA CTG TTA ATC AGC TGG CCC-3′ (sense) and 5′-GGT CAT CTC TCA AGA GGG CA-3′ (antisense), *Mfn2*—5′-ATG CTT CCC CTC TCA AGC AC-3′ (sense) and 5′- GCT CTC TTG GAT GTA GGC CC-3′ (antisense), *Opa1*—5′-ACG GGT TGT TGT GGT TGG AG-3′ (sense) and 5′-GTG TCA TCA TCT CGC CGG AC-3′ (antisense), *Oma1*—5′-GGG CAG GGG CAT AAG GAA AT-3′ (sense) and 5′-ACT CAG ACC AAG AAG CAG CC-3′ (antisense), and *Dnm1l*—5′-GTA AGC CCT GAG CCA ATC CA-3′ (sense) and 5′-

Table 1: Primary and secondary antibodies used for Western blotting.

Antibody	Dilution	Order number
Anti-MFN1 mouse monoclonal IgG antibody	1 : 100 in Roti-TBS (#1060.1, Roth, Germany)	#sc-166644, Santa Cruz, USA
Anti-MFN2 mouse polyclonal IgG antibody	1 : 100 in Roti-TBS (#1060.1, Roth, Germany)	#ARP89255_P050, Aviva systems biology, USA
Anti-OPA1 mouse monoclonal IgG antibody	1 : 100 in Roti-TBS (#1060.1, Roth, Germany)	#sc-393296, Santa Cruz, USA
Anti-OMA1 mouse monoclonal IgG antibody	1 : 100 in Roti-TBS (#1060.1, Roth, Germany)	#sc-515788, Santa Cruz, USA
Anti-Drp1 mouse monoclonal IgG antibody	1 : 500 in Roti-TBS-T (#1061.1, Roth, Germany)	#14647, Cell Signaling, USA
Anti-pDrp1 rabbit monoclonal IgG antibody	1 : 500 in Roti-TBS-T (#1061.1, Roth, Germany)	#4494, Cell Signaling, USA
Anti-CaMKII (pan) rabbit polyclonal antibody	1 : 500 in Roti-TBS-T (#1061.1, Roth, Germany)	#3362, Cell Signaling, USA
Anti-ox-CaMKII rabbit polyclonal antibody	1 : 500 in Roti-TBS-T (#1061.1, Roth, Germany)	#07-1387 Merck, Germany
Anti-calnexin rabbit polyclonal IgG antibody	1 : 200 in Roti-TBS (#1060.1, Roth, Germany)	#sc-11397, Santa Cruz, USA
Anti-actin rabbit polyclonal IgG antibody	1 : 1000 in Roti-TBS (#1060.1, Roth, Germany)	#A2668; Merck, Germany
Anti-rabbit goat horseradish-peroxidase-conjugated antibody	1 : 10.000 in Roti-TBS (#1060.1, Roth, Germany)	#sc-2054, Santa Cruz, USA
Anti-mouse donkey antibody	1 : 10.000 in Roti-TBS (#1060.1, Roth, Germany)	#sc-2314, Santa Cruz, USA

CTC GAT GTC CTT GGG CTG AT-3′. Melting curves were recorded after each cycle and showed individual PCR products. Expression levels of the genes of interest and the housekeeping genes were measured in triplicate in three independent PCR runs. The collected data were analyzed using the $2^{-\Delta\Delta CT}$ method [37]. GraphPad Prism 7 software (GraphPad Software, USA) was used for data evaluation. The data are presented as the mean values ± SEM. The Kolmogorov-Smirnov normality test was used to confirm normal distribution. Student's *t*-test was performed for significance testing between the two genotype groups, and values with $p < 0.05$ were considered to be significant.

2.9. SDS Gel Electrophoresis and Western Blotting. For Western blotting, proteins from cervical spinal cord were isolated using cell lysis buffer (#9803S, Cell Signaling Technology, USA) supplemented with protease inhibitor (#11697498001, Merck, Germany). To determine protein concentrations, Pierce™ BCA Protein Assay Kit (#23225, Thermo Fisher Scientific, Germany) was used. 50 μg of total protein was separated by SDS gel electrophoresis and transferred to a nitrocellulose membrane. Subsequently, the blots were blocked by incubation in 1% RotiBlock (#A151, Roth, Germany) in phosphate-buffered saline (PBS) for at least 1 h at room temperature. Primary antibodies (Table 1) were incubated overnight at 4°C. HRP-coupled secondary antibodies (Table 1) were incubated for 1 h at room temperature. Finally, Immuno Cruz Luminol Agent (#sc-2048, Santa Cruz Biotechnology, USA) was used for signal detection with an imaging system (ChemiDoc XRS+, BioRad, USA). For arithmetic analysis of the band intensity, ImageJ 1.51s (National Institutes of Health, USA) software was used. Band intensities of interested proteins were normalized to the housekeeper calnexin or actin. Normalized protein levels were compared between different genotypes. Data analyses were performed using GraphPad Prism 7 software (GraphPad Software, USA). The results are presented in bar charts with the respective percentage. Data were reported as normalized means ± SEM. The Kolmogorov-Smirnov normality test was used to confirm normal distribution. Student's *t*-test was performed for significance testing between the two genotype groups, and values with $p < 0.05$ were considered to be significant.

3. Results

In numerous studies on neurodegenerative diseases, altered mitochondria were a common phenomenon observed in the affected cells [38–40]. In the pathogenesis of Alzheimer's disease, for example, a reduction in the number and size of mitochondria has been identified as a key step in pathogenesis [41]. Especially in ALS, mitochondria seem to play a crucial role in the degeneration of mitochondrially highly active motor neurons [20]. Based on our recent findings of elevated ROS levels in wobbler mice [31], our interest was focused on motor neuronal mitochondria in this study. The purpose of this study was to reveal mitochondrial abnormalities in motor neurons of wobbler mice and to provide evidence for possible pathological underlying mechanisms.

3.1. Disturbed Mitochondrial Network in Wobbler α-Motor Neurons. We used dissociated cell cultures and immunofluorescent staining to visualize the mitochondrial network in the soma of motor neurons. Therefore, we cultivated dissociated motor neuronal enriched cultures of wild-type (WT) and wobbler diseased (WR) mice for 10 days. We compared the mitochondrial network of 50 motor neurons from WT and WR of three independent preparations with aid of superresolution microscopy with structured illumination in combination with the analysis software Imaris 9.2.1. We used the spots and surface function to compare the MitoTracker signal and thereby the mitochondrial network (Figure 1(a)). The surface function enables the study of mitochondrial chains and thus the continuity of the mitochondrial network. The spot function rather represents higher local intensities within the network or single mitochondria. Obtained parameters were quantitatively evaluated. The area, volume, and diameter of spots and thus single mitochondria were significantly diminished in homozygous wobbler mice (Figure 1(b)). Surface function showed a trend towards smaller mitochondrial chains in motor neurons from diseased mice, due to a slightly smaller surface area and volume (Figure 1(c)). By evaluating the mitochondrial chains/surfaces, we did not find any clear differences in the ellipsoid axes. But we were able to detect strong differences in MitoTracker signal intensity in the motor neuronal soma. Both the mean and the maximum intensity of MitoTracker signal were significantly increased for the measured wobbler spots and surfaces compared to WT in the region of interest (Figures 1(b) and 1(c)). This could be related to an accumulation of single mitochondria, resulting in stronger MitoTracker signals per area.

3.2. Smaller and Irregularly Shaped Mitochondria of Wobbler Mice Motor Neurons. Since our immunofluorescence investigations showed evidence of smaller spots in the mitochondrial network, we aimed to clarify whether this might be reflected in detail by morphologically abnormal mitochondria. Therefore, transmission electron microscopy (TEM) of the cervical spinal cord of WT and WR mice was performed (Figure 2(a)). Quantitative and qualitative analyses of specific characteristics revealed abnormal mitochondrial morphology in motor neurons of WR mice (Figure 2(b)). The analysis revealed that WR mitochondria are significantly smaller than WT mitochondria. In addition, a significant decrease in roundness, circularity, and perimeter was identified in WR mitochondria compared to WT mitochondria. Aspect ratio was significantly increased in WR compared to wild-type littermates. No clear differences could be found in Feret's diameter. Counting mitochondria revealed significant more mitochondria per motor neuron in wobbler mice. Altogether, smaller and elongated mitochondria were detected in motor neurons of wobbler mice at the stable phase of the disease (p40) compared to wild-type mice using TEM. Qualitative observation revealed internal vacuolization, misfolded inner mitochondrial membrane (IMM), and altered crista structure in most mitochondria in WR motor neurons, indicating degeneration of these organelles. In contrast, we observed larger mitochondria with intact IMM in motor neurons at the time of the first appearance of symptoms (p20) in the wobbler mice compared to wild-type (Figure S1).

3.3. 3D Visualization of the Mitochondrial Crista Structure. Since evidence of a misfolded IMM was considered by TEM in p40 motor neurons of mitochondria, we visualized this in more detail by TEM tomography. The aim was to reveal possible differences in crista junctions or crista shape of the inner mitochondrial membrane. Motor neurons were identified by their specific characteristics, and mitochondria were selected analogous to our prior TEM investigations. TEM tomography supported the assumption derived from our TEM studies suggesting a degenerated and misfolded IMM as shown in Figure 3. In addition to reduced mitochondrial area and its irregular shape in WR motor neurons observed by TEM, TEM tomography revealed strong indications for a reduction of the IMM area in WR motor neurons. Moreover, the number of cristae and thus crista junctions per mitochondrion might be reduced compared to wild-type mitochondria (Figure 3). Additional movie files show a reconstruction of the z-stack of a wild-type and wobbler mitochondrion (Movie S1) and videos of the 3-dimensional model of these mitochondria (Movie S2).

3.4. Abnormal Fission-Related Proteins in the Cervical Wobbler Spinal Cord. Since our data demonstrated an abnormal mitochondrial morphology in motor neurons of the cervical spinal cord of wobbler mice, the question arose whether altered mitochondrial dynamics are present in wobbler mice at the stable phase of the disease. Here, we found that all investigated mRNA levels of fusion-related proteins *Mfn1* and *Mfn2*, *Opa1*, and *Oma1* were significantly reduced in cervical spinal cord of WR mice (Figure 4(a)). In order to verify these findings on a protein level, Western blots were performed (Figure 4(b)). It was not possible to confirm the expression differences found on mRNA level. On the contrary, Mfn2 protein expression was found to be significantly increased in WR motor neurons. On the site of fission related proteins, we took a closer look at the expression of the gene *Dnm1l* and the corresponding Drp1 protein. The expression of total Drp1 did not differ between WT and WR, neither on mRNA nor on protein level (Figure 4(c)). We further investigated important mechanisms influencing the activity of Drp1. In order to do so, we first investigated the phosphorylation status of the Drp1 at Ser616, as this phosphorylation is associated with increased Drp1 activity. Our investigations discovered that a significant larger proportion of Drp1 was phosphorylated at Ser616 in wobbler spinal cord in contrast to WT (Figure 4(c)). Furthermore, we could show that the Ca2+/calmodulin kinase (CaMKII), an important posttranslational regulator of Drp1, was significantly more abundant in its oxidized and thus activated form in WR motor neurons compared to WT (Figure 4(d)).

4. Discussion

It is well known that oxidative stress in motor neurons leads to massive impairments and degenerative changes [42, 43].

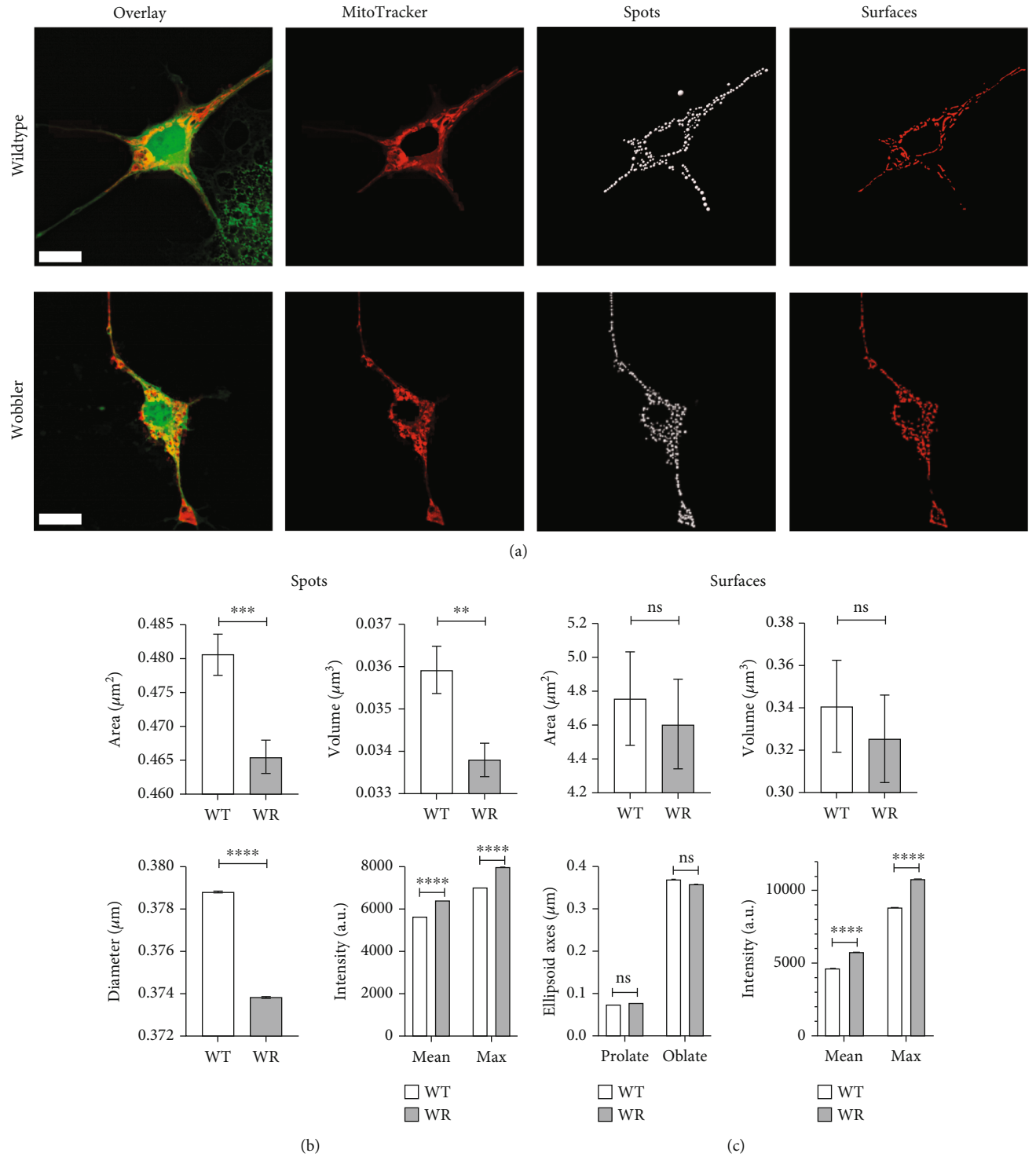

Figure 1: Disturbed mitochondrial network in wobbler α-motor neurons. (a) Motor neuron enriched cultures of p40 WT and WR after 10d *in vitro*. Staining was performed with CellTracker (green) and MitoTracker (red). The mitochondrial network was reconstructed and quantitatively analyzed with Imaris 9.2.1 (b) spots and (c) surface function. Due to a significant decrease in area, volume, and diameter of spots as well a slightly diminished surface area and volume, a disturbed, fragmented mitochondrial network is present in wobbler motor neurons. No clear differences could be found in surface ellipsoid axes. Mean and maximum MitoTracker intensity of spots and surfaces are significantly higher in wobbler motor neurons, probably explained by more individual, smaller mitochondria per area. Data are presented as means ± SEM. For significance testing, students t-test was performed. Significant differences are indicated by ns > 0.05, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Scale bar = 10 μm (a). A total of 50 motoneurons from four independent preparations per genotype were examined. n(spots) = 10415-11815; n(surfaces) = 1290-1433.

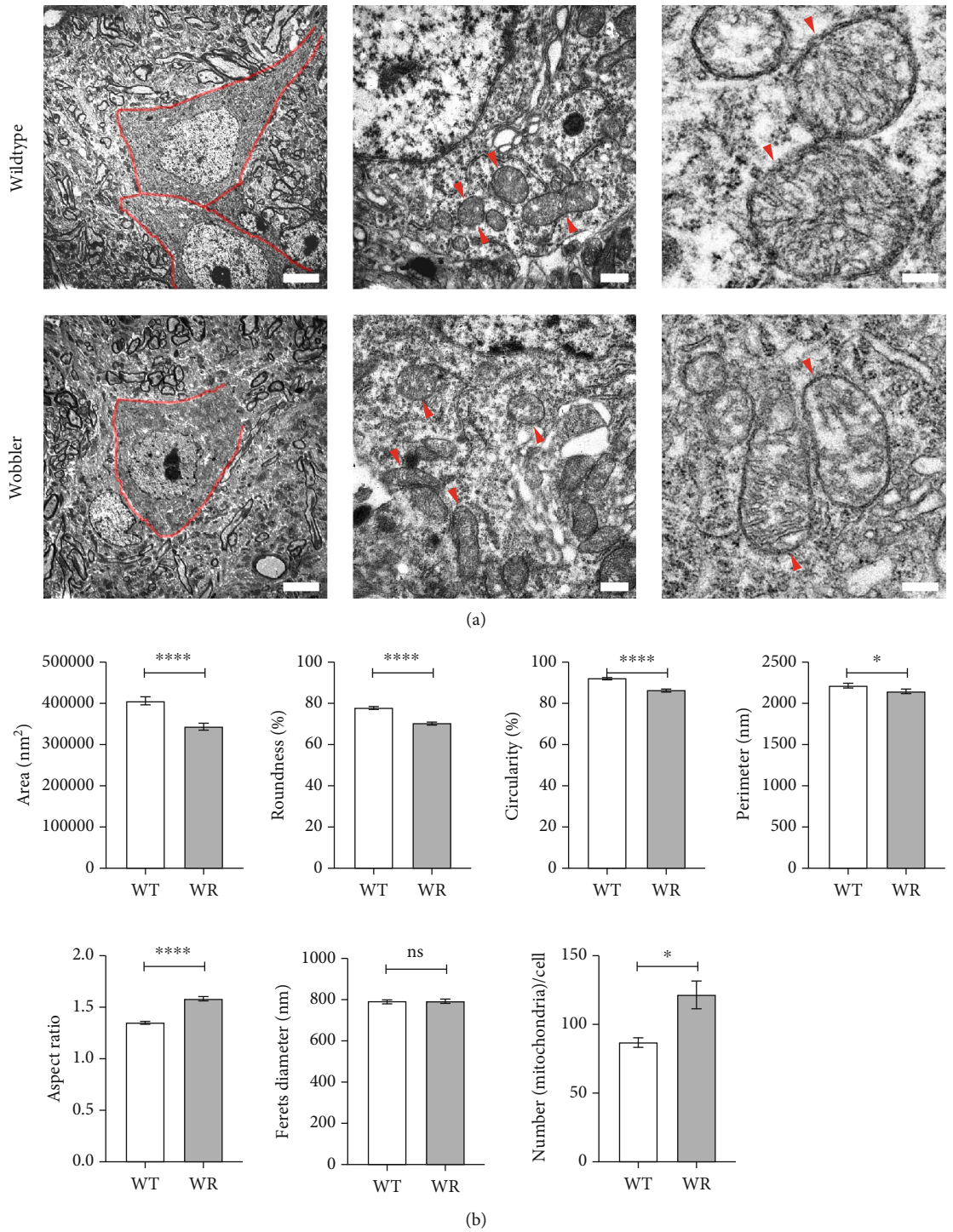

Figure 2: Smaller and irregularly shaped mitochondria of wobbler mice motor neurons at p40. Transmission electron microscopy of cervical spinal cord of wild-type and wobbler mice at p40. (a) Overview images of motor neurons (red border) and magnified single mitochondria (red arrowheads), indicating an altered mitochondrial morphology and degeneration of crista structure in wobbler mice. (b) Measurement of mitochondria with ImageJ revealed a significant decrease in area, roundness, circularity, and perimeter combined with a significantly increased aspect ratio in wobbler mice. No differences in Feret's diameter could be detected. Counting mitochondria demonstrated an increase of mitochondrial number per motoneuron. In summary, smaller, irregularly shaped, and elongated mitochondria are present in wobbler motor neurons at p40. Data are presented as the means ± SEM. For significance testing, Student's t-test was performed. Significant differences are indicated by ns > 0.05, $^{*}p < 0.05$, and $^{****}p < 0.0001$. Scale bar = 5 μm (left), 500 nm (middle), and 200 nm (right). $N = 5$; $n = 1066$-1092 mitochondria per genotype.

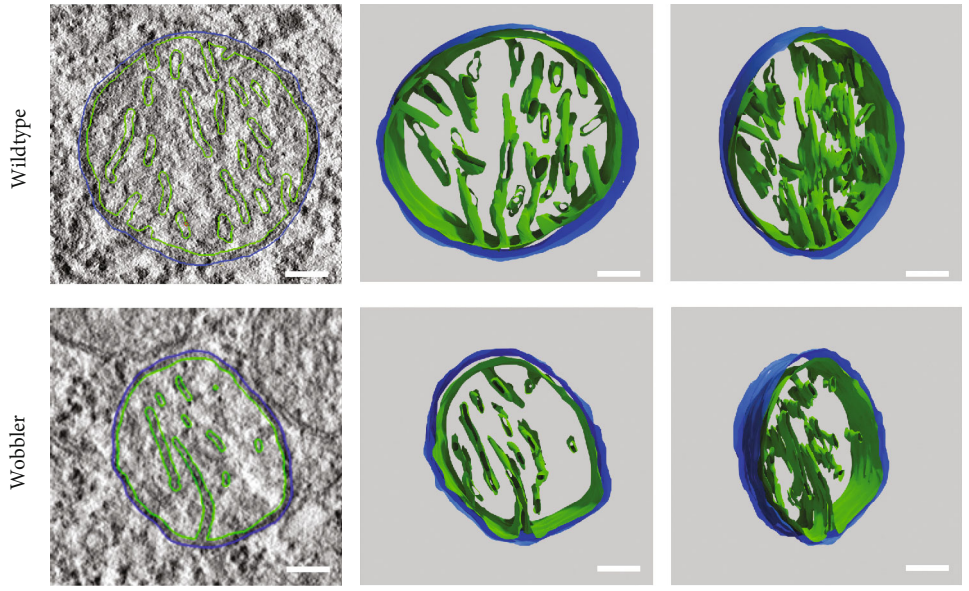

Figure 3: Three-dimensional visualization of the mitochondrial crista structure. TEM tomography of cervical spinal cord of wild-type and wobbler mice. (a) Exemplary image of a recorded plane of TEM tomography with reconstruction of mitochondrial membranes. Blue line represents the modulation of the outer mitochondrial membrane (OMM), while the green line modulates the inner mitochondrial membrane (IMM). (b) Front view and tilted view of the finally meshed 3D models of WT and WR mitochondria. Models imply a misfolded, smaller IMM and a reduction in cristae as well as crista junctions. $N = 2$. Scale bar = 100 nm.

In various neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, autosomal-dominant optic neuropathy, and even some human ALS cases, several defects in mitochondrial function, dynamics, and ROS production, leading to oxidative stress and thus degeneration, have been identified in causal relations [38]. This underlines the importance of healthy mitochondria for the maintenance of neuronal structures. Our previous studies have been able to uncover strongly increased ROS levels and indications for defects in antioxidant capacity in the spinal cord of wobbler mice, an ALS animal model, at the stable phase of the disease [26, 31]. The purpose of this study was to reveal morphological differences in mitochondrial architecture and network. It is questionable whether impaired mitochondria are additionally leading to high ROS values or if impaired mitochondrial function and morphology are a consequence of increased ROS levels. Based on this question, we aimed to find possible primary causes of mitochondrial changes that would explain the previously found elevated ROS levels. Finally, this work should clarify whether there are common pathomechanisms with other ALS models and human ALS cases. This would possibly provide new therapeutic targets from which a larger cohort of patients could benefit in the future.

Mitochondria provide the energy supply needed by the machinery of every living cell. The majority of mitochondria are found in the cell soma. Furthermore, processes responsible for the degradation of damaged mitochondria by autolysosomes also predominantly take place in this area. For this reason, the cell soma is particularly important for the detection of increased mitochondrial degradation [44], and we therefore focused on the characterization of mitochondria in this region.

In this study, we showed that the mitochondrial network of wobbler motor neurons from p40 mice is split into more individual, smaller, single components in comparison to the wild-type mitochondrial network. This is represented by a similar size of the reconstructed surfaces and a reduction in the spot size. We assume that these individual, smaller components are accumulating, explaining the strong increased MitoTracker signal in our immunofluorescence investigations. We were also able to show on ultrastructural level smaller and elongated mitochondria due to a decrease in perimeter and size as well as an increase in aspect ratio and number of mitochondria per motoneuron in stable diseased mice. This is combined with an irregular shaped morphology of mitochondria through a diminished roundness of wobbler mitochondria in motor neurons compared to wild-type. By 3D reconstruction of a mitochondrial section with TEM tomography, we confirmed further endorsement for these differences and additionally identified strong indications for a misfolded, smaller inner mitochondrial membrane, and reduced cristae in combination with less crista junctions in WR motor neurons in comparison to wild-type. In contrast to the late diseased stage in wobbler mice at p40, the occurrence of the first ALS typical symptoms at p20 went along with larger and rounder mitochondria in the motor neurons of the cervical cord without signs of degeneration of the IMM. Others described the mechanism of fusion with a neighboring healthy mitochondrion as a process attempting to mitigate an existing damage, thus enabling to mix mitochondrial DNA, proteins, lipids, and

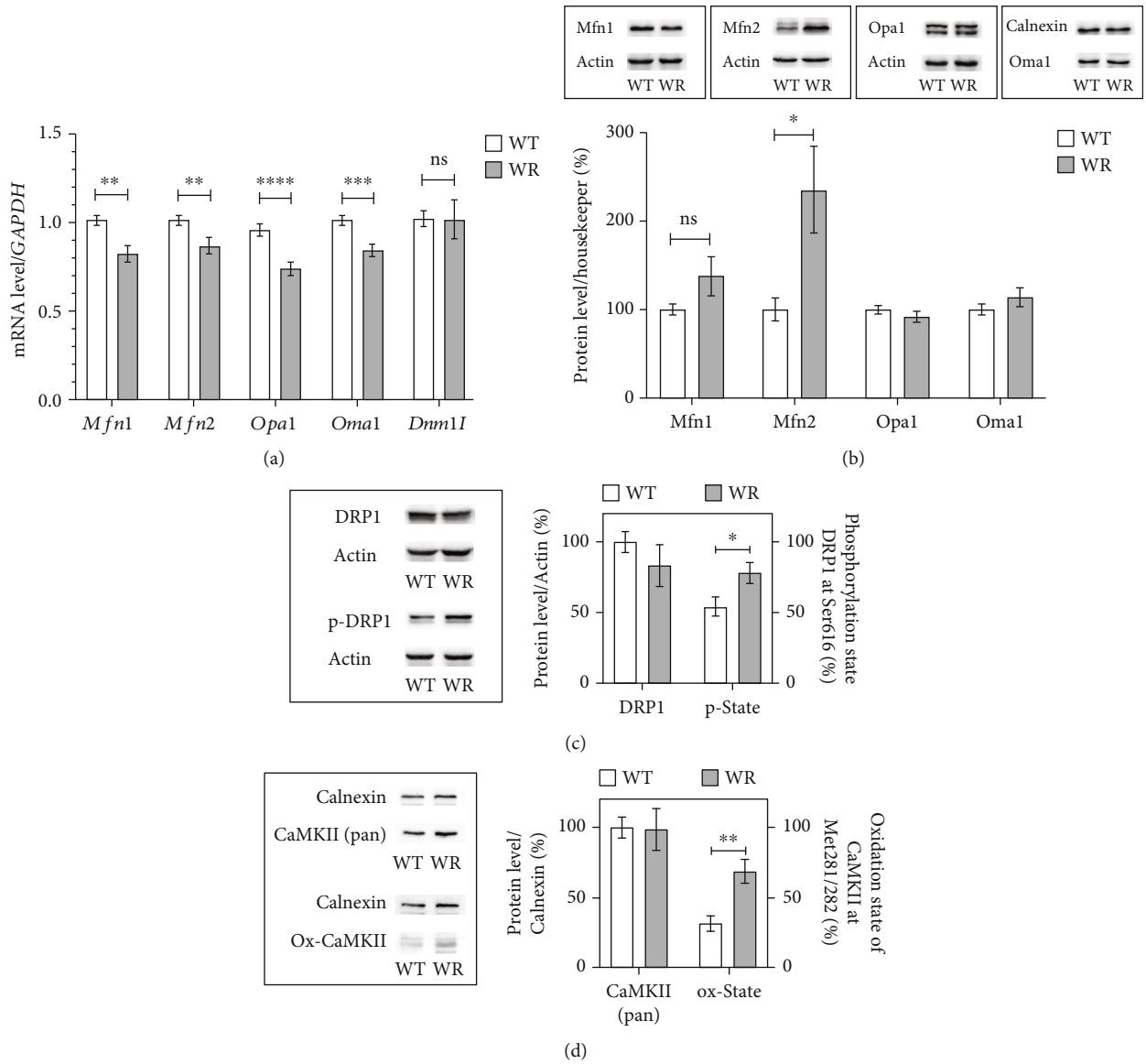

Figure 4: Abnormal fission-related proteins in cervical wobbler spinal cord at p40. (a) mRNA expression levels of *Mfn1*, *Mfn2*, *Opa1*, *Oma1*, and *Dnm1l* from the stable phase of wild-type (WT) and wobbler (WR) spinal cords were investigated by qPCR. mRNA levels were significantly reduced in WR except *Dnm1l*. For relative quantification, the $2^{-\Delta\Delta Ct}$ method was conducted using *GAPDH* for normalization. $N = 7$-11 per genotype. (b) Exemplary Western blots of Mfn1 (≈85 kDa), Mfn2 (≈85 kDa), Opa1 (≈100 kDa), and Oma1 (≈50 kDa) in the cervical spinal cord of p40 WT and WR. Actin (≈45 kDa) and calnexin (≈90 kDa) were used as control proteins. Bar charts represent the semiquantitative analysis of protein expression levels. Western blots revealed unchanged expression of Mfn1, Opa1, and Oma1 as well as significantly increased expression of Mfn2 in the cervical spinal cord of wobbler mice. $N = 8$-10 per genotype. (c) Exemplary Western blots of Drp1 (≈85 kDa), p-Drp1 (Ser616; ≈85 kDa), and actin (≈45 kDa) as control protein. Analysis of band intensity is presented in bar charts. Total amount of Drp1 does not differ between the two genotypes; Drp1 is significant more often phosphorylated at Ser616 in cervical spinal cords of wobbler mice. $N = 9$ per genotype. (d) Exemplary Western blots of CaMKII (≈55 kDa) and oxidized Ox-CaMKII (Met281/282; ≈55 kDa) in combination with calnexin (≈90 kDa) as control protein from wild-type and wobbler cervical spinal cords. Analysis of band intensity is presented in bar charts and showed equal CaMKII levels; thus, the proportion of oxidized CaMKII at Met281/282 is significantly increased in wobbler spinal cords compared to wild-type. $N = 7$ per genotype. All data are presented as the mean values ± SEM, and Student's t-test was performed for significance testing between WT and WR. Values with $p < 0.05$ were considered to be significant. Significant differences are indicated by ns > 0.05, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

metabolites [45–47]. However, in case the mitochondrial membrane potential cannot be maintained and the organelle is severely damaged, a mitochondrial fusion process cannot occur to maintain mitochondrial integrity. Thus, the cell implies mitochondrial fission processes to generate small mitochondria that can be more easily degraded [48–51]. Our results indicate the existence of such a process within the motor neurons of wobbler mice. Here, the assumption

arises that at stage p20, mitochondria are still undergoing fusion to minimize damage that has occurred. Since we did not detect increased ROS levels in the cervical spinal cord in a previous study at p20 [31], another stressor must be involved as a trigger of mitochondrial damage. Another hypothesis would be that more ROS are already produced in motor neurons at p20 compared with wild-type mice, while antioxidant mechanisms still function sufficiently to counteract this imbalance. Thus, further studies are needed to identify or verify the actual stressor that leads to mitochondrial damage early in the disease. This stressor will likely still be present after p20 and continue to damage mitochondria, so fusion will not mitigate mitochondrial damage over the long term. The mitochondria cannot maintain their membrane potential, and the cells are more likely to undergo fission, as we see in the motor neurons of wobbler mice at stage p40. Furthermore, it is known that in diseases associated with decreased mitochondrial respiratory chain function, as it appears to be the case in ALS [20] and wobbler mice [27–30], decreased mitochondrial membrane potential is associated with an increase in ROS production at mitochondria [28, 52].

A fragmentation of the mitochondrial network to smaller single components has already been described in many neurodegenerative diseases including some subtypes of ALS [38, 53]. Ultimately, these cases could be attributed to an imbalance in fusion-fission activity. For this reason, we initially suspected that a restricted fusion activity or increased fission activity could be causative for the fragmentation at the stable phase of the wobbler disease. Therefore, we investigated the expression of fusion and fission-related proteins. Here, we could not find any differences in the expression of fusion-related proteins that would explain the described morphology of the mitochondria and the deconstructed mitochondrial network in the motor neurons of diseased animals at p40. Strikingly, the expression of the fusion-related protein Mfn2 was increased in the cervical spinal cord of wobbler animals. Previous studies could only attribute fragmentation of the mitochondrial network to a downregulation of Mfn2 [54]. In these cases, fragmentation subsequently led to increased glutamate-induced excitotoxicity and neuronal cell death. On the contrary, it has been shown that upregulation of Mfn2, as we could show in wobbler tissue, has a protective effect against these events [55]. In particular, considering that glutamate-induced excitotoxicity plays a central role in the pathophysiology of ALS [56], Mfn2 upregulation could be interpreted as a compensatory mechanism for the cell to protect itself from glutamate-induced excitotoxicity. Interestingly, an overexpression of Mfn1 has been found in *C9Orf72*-ALS/FTD patient fibroblasts in combination with mitochondrial fragmentation as well [57]. There is some debate whether this is a compensatory mechanism [20]. More detailed studies are needed to confirm this mechanism, as it is not fully understood to date.

Studies in neurodegenerative Parkinson's disease reveal an overexpression of the fission related protein Drp1. This caused segmentation of the mitochondrial network into smaller components and led to neuronal degeneration [58]. Although the mitochondrial network described in these Parkinson cases is very similar to the mitochondrial network found in the motor neurons of our wobbler mice, we could not confirm a Drp1 dysregulation in our study. Our analyses of the fission-related protein revealed no abnormalities at the mRNA or at the protein level. For this reason, dysregulated mitochondrial fission initially seemed to be unsuitable as a reason for the segmentation.

Increased oxidative stress leads to numerous damages in the cell, some of which can further multiply the oxidative stress [59]. An elevated ROS level in the spinal cord of wobbler mice could lead to DNA damage, lipid oxidation, protein oxidation and aggregation, induction of inflammatory processes, excitotoxicity, reduction in the efficiency of cellular process, and apoptosis [20, 60, 61]. Mitochondrial DNA is particularly sensitive to ROS since mitochondria have limited DNA repair mechanisms [20]. Elevated ROS levels are frequently described and discussed in the context of ALS (reviewed in 63). Even in biofluid samples and postmortem tissue biopsies from ALS patients [62, 63], elevated biomarkers for oxidative stress have been detected. Additional consequences of oxidative stress were studied in $SOD1^{G93A}$ transgenic mice, a mouse model for familiar form of ALS, which are summarized in Barber and Shaw's study [64]. In this context, it must be emphasized that oxidative stress leads to aggregation of SOD1 [65], which induces mitochondrial dysfunction [66]. This finally ends in a self-reinforcing process with fatal consequences, as mitochondrial dysfunction further promotes free radical production and thus misfolding of SOD1 [20].

However, it is known that many kinases and proteases carry an oxidizable cysteine residue acting as a sensor for activation, as in case of Ca2+/calmodulin kinase II (CaMKII) [67]. Here, we show that the expression of CaMKII protein does not differ overall between WT and WR, although the proportion shifted significantly towards the oxidized form (Ox-CaMKII) in homozygous wobbler mice in comparison to wild-type animals. Recent studies in triple negative breast cancer cells have shown that oxidation of CaMKII to Ox-CaMKII directly leads to increased phosphorylation of Drp1 at the Ser616. This breast cancer study further revealed that CaMKII is activated by all kind of ROS, especially superoxide anions [68]. In this context, it is interesting to note that impaired functions of complexes I and III of the mitochondrial electron transport chain, as present in the cervical spinal cord of wobbler mice, lead to elevated superoxide levels by electron efflux and spontaneous oxidation of molecular oxygen [28]. In addition to mitochondrial dysfunction, increased activation of microglial cells may increase the expression of NADPH oxidase, which physiologically produces further superoxide anion [13, 64]. Against this background, it is interesting that Dahlke et al. [23] also observed microglial activation in the cervical spinal cord of wobbler mice, probably promoting NADPH expression and thus superoxide anion production. We assume that impaired mitochondrial function in combination with activated microglia causes an increase in superoxide anion production, leading to oxidation of CaMKII at the stable phase of the disease in wobbler mice.

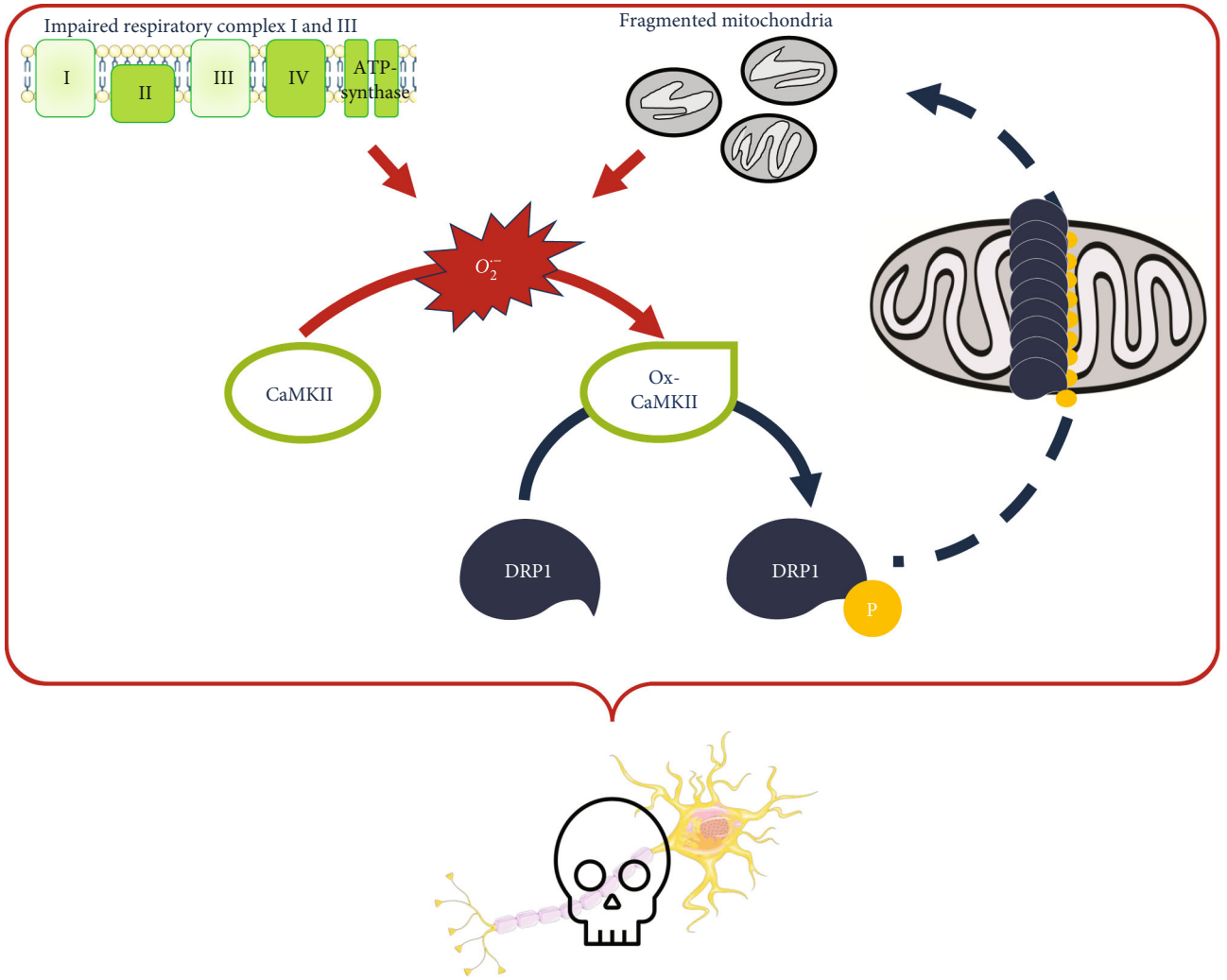

Figure 5: Proposed mechanism of motor neuronal cell death in wobbler mice. An impaired function of complexes I and III of the mitochondrial respiratory chain leads to an increase in superoxide anions. Increased superoxide anion levels cause an oxidation and thus calcium-independent activation of CaMKII. Ox-CaMKII in turn stimulates phosphorylation of Drp1 at Ser616, which recruits it to the mitochondrial membrane and causes enhanced mitochondrial fission. This disruption between fusion and fission balance promotes fragmentation of the mitochondrial network, resulting in increased production of reactive oxygen species. This is likely to trigger a nonreversible process that leads to fragmented and dysfunctional mitochondria, resulting in a self-reinforcing vicious circle that promotes degeneration of motor neurons in wobbler mice.

Crucial for the cell are the consequences of CaMKII activation. Calcium-independent activation of CaMKII by methionine oxidation leads to increased fission activity, as it is associated with phosphorylation of Drp1 at the Ser616 and thus increased recruitment of Drp1 to the mitochondrial membrane and fission of mitochondria [69]. Based on our results, the mechanism of ROS-regulated, Ox-CaMKII-dependent Drp1 activation may also play a role in wobbler motor neurons. We showed that Drp1 is significantly more frequently phosphorylated at Ser616 in the cervical spinal cord of diseased animals compared to WT. This could finally explain an increased fission of mitochondria in wobbler motor neurons, ultimately leading to fragmentation of the mitochondrial network into more individual, smaller components. In studies on myocardial ischemia, Drp1-dependent fragmentation of the mitochondrial network was also observed [70]. They discovered that Drp1-dependent fragmentation of the mitochondria increased mitochondrial ROS production. The increased ROS production then further increases mitochondrial fragmentation in terms of a vicious circle [71, 72]. Given our findings of fragmented mitochondria due to Drp1 activation in motor neurons and increased ROS in the spinal cord of wobbler mice at p40, this mechanism might also be present in the wobbler animal model. Fragmented mitochondria have immense consequences for cell physiology. Studies show that Bax and Bak, both proapoptotic molecules, interact with activated Drp1 and thereby initiate apoptosis. Fragmented mitochondria are therefore an early indicator for cell death [40]. In cultured motor neurons of mutant $SOD1^{G93A}$ mice, mitochondrial fragmentation was

also observed and directly linked to reduced axonal transport and neurite length and even increased cell death [73]. It is further suggested that fragmented mitochondria are linked to lower ATP levels, reduced mitochondrial membrane potential, and energetically poorer function [20, 74].

Nevertheless, it is still difficult to decide whether increased ROS levels cause fission, leading to dysfunctional mitochondria, or whether dysfunctional mitochondria promote ROS production, leading to increased fission. The outcome of both events is the same. However, since we already detect abnormal mitochondrial morphology at p20 without detecting elevated ROS levels, mitochondrial damage not primarily triggered by oxidative stress can be assumed. Thus, oxidative stress does not seem to be the main factor for motor neuron damage at an early stage of the disease, but a consequence of mitochondrial dysfunction at later stages of wobbler disease. Another point confirming mitochondria as the reinforcing factor of mitochondrial fission in p40 wobbler animals is the fact that impaired mitochondria are the main producers of superoxide anions, which are most important for Ox-CaMKII-dependent Drp1 activation. Moreover, the purpose of Drp1 activation remains partially unclear, as it continuously drives the cell into an apoptotic metabolic state. One possible explanation represents an automechanism by which the cell shuts itself down to prevent a catastrophic event. Another explanation could be that fragmentation causes the degradation of defective mitochondria so that the cell maintains healthy and efficient mitochondrial structures [75]. Further, it was found that Mfn1 or Mfn2 overexpression is able to counteract mitochondrial fragmentation and even attenuate cell death [76]. This would support the hypothesis that increased expression of Mfn2, as we have demonstrated in our study in wobbler spinal cord, is a possible attempt to provide a rescue mechanism of motor neurons from cell death. However, further studies on fission and fusion processes in regard to the interplay between ROS, Drp1, and Mfn2 in motor neurons are needed to gain a full understanding of the underlying mechanism and its potential on motor neuronal degeneration.

5. Conclusions

The present study focused on the morphological analysis of the mitochondrial network and individual mitochondria in motor neurons of wobbler mice, an ALS animal model, in context of previous studies discovering elevated ROS levels in wobbler spinal cords at the stable phase of the disease (p40). We were able to identify several pathologies in wobbler motor neurons, like a fragmented mitochondrial network, consisting of more individual, accumulated mitochondria. We suspect that a reduced function of complexes I and III leads to an increase in superoxide anions, which is maybe further enhanced by activated microglia. Increased superoxide anion levels cause an oxidation and thus calcium-independent activation of CaMKII. Ox-CaMKII in turn stimulates phosphorylation of Drp1 at Ser616, which recruits it to the mitochondrial membrane and causes enhanced mitochondrial fission. Finally, this disrupts the balance between fusion and fission and promotes fragmentation of the mitochondrial network, resulting in increased production of reactive oxygen species (Figure 5). Under physiological circumstances, fission ensures a depletion of dysfunctional mitochondria and maintaining homeostasis. But in context of some ALS cases and probably wobbler disease, mitochondrial fission takes over. This is likely to trigger a nonreversible process that enhances fragmented and dysfunctional mitochondria, resulting in a self-reinforcing vicious circle that additionally promotes degeneration. Nowadays, treatment of ALS patients with Edaravone, the first antioxidant compound for the treatment of ALS that counteracts the process of oxidative stress, reveals promising results, prolonging life by several months. However, since we observe signs of damage in motor neuronal mitochondria at an earlier stage of the disease without evidence of oxidative stress, the search for causative factors should continue. Furthermore, no curative therapeutic approach has been found to date, so further studies are required to cure this fatal disease one day.

Data Availability

All data generated in this study are published in this article and its supplementary files.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Figure S1 (separate file). Larger and Rounder Mitochondria of Wobbler Mice Motor Neurons at p20. Transmission electron microscopy of cervical spinal cord of wild-type and wobbler mice at p20. (A) Overview images of motor neurons (red border) and magnified single mitochondria (red arrowheads), indicating an altered mitochondrial morphology in wobbler mice. (B) Measurement of mitochondria with ImageJ revealed a significant increase in area, roundness, circularity, perimeter, and Ferrets diameter combined with a significantly decreased aspect ratio in wobbler mice. Counting mitochondria demonstrated no significant alteration in mitochondrial number per motoneuron. In summary, larger and rounder mitochondria are present in wobbler motor neurons at p20. Data are presented as the means ± SEM. For significance testing, Student's t-test was performed. Significant differences are indicated by ns > 0.05, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$. Scale bar = 5 μm (left),

500 nm (middle), and 200 nm (right). $N = 3$; $n = 750$ mitochondria per genotype. Movie S1 (separate file). TEM tomography of motor neuronal mitochondria of wild-type and wobbler mice. Exemplary video of different planes of reconstructed z-stack of a wild-type and a wobbler mitochondrion. Scale bar = 100 nm. Movie S2 (separate file). Three-dimensional model of motor neuronal wild-type and wobbler mitochondrion. Image alignment and reconstruction by filtered back projection were carried out using the software package IMOD 4.9.7. A 3D model was generated by manual segmentation of the reconstructed image stack with the segmentation feature of 3dMod from the IMOD package. On each image plane, individual objects with corresponding contours specific to the mitochondrial structures were assigned and exported as a surface mesh. Small mismatches and failures in the mesh were corrected with the software MeshLab 2020.07 (Institute of Information Science and Technology, Italy), and the final result was rendered by a raytracing algorithm implemented in the software Blender 2.83.2 (Blender Foundation, Netherlands). *(Supplementary Materials)*

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Review Article

The Critical Role of Oxidative Stress in Sarcopenic Obesity

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Sarcopenic obesity (SO) is a combination of obesity and sarcopenia that primarily develops in older people. Patients with SO have high fat mass, low muscle mass, low muscle strength, and low physical function. SO relates to metabolic syndrome and an increased risk of morbimortality. The prevalence of SO varies because of lacking consensus criteria regarding its definition and the methodological difficulty in diagnosing sarcopenia and obesity. SO includes systemic alterations such as insulin resistance, increased proinflammatory cytokines, age-associated hormonal changes, and decreased physical activity at pathophysiological levels. Interestingly, these alterations are influenced by oxidative stress, which is a critical factor in altering muscle function and the generation of metabolic dysfunctions. Thus, oxidative stress in SO alters muscle mass, the signaling pathways that control it, satellite cell functions, and mitochondrial and endoplasmic reticulum activities. Considering this background, our objectives in this review are to describe SO as a highly prevalent condition and look at the role of oxidative stress in SO pathophysiology.

1. Introduction

Sarcopenic obesity (SO) was described in 1996 by Heber et al. [1], but it is not clearly defined. Nevertheless, the most accepted definition of SO is a combination of obesity and sarcopenia, mainly, although not exclusively, in older people. SO is characterized by high fat mass, low muscle mass, low muscle strength, and low physical functionality [1–7]. People that develop SO are primarily over 60 years old, with comorbidities such as type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver diseases (NAFLD), dyslipidemia, hypertension, and cardiovascular disease. They generally have a sedentary lifestyle and engage in harmful habits such as tobacco and alcohol consumption and a high-fat and/or carbohydrate diet [3].

SO associates with a high risk of hospitalization, loss of independence, disability, frailty, increased risk of fractures, impaired quality of life, higher mortality, and multimorbidity [8–12]. Thus, SO decreases the physical functional capacity to a higher degree than sarcopenia or obesity separately

[13, 14]. Considering this background, SO is regarded as a severe public health problem.

SO relates to metabolic syndrome (hypertension, hyperglycemia, insulin resistance (IR), T2DM, abnormal lipid metabolism, and dyslipidemia) and lower cardiorespiratory fitness [11, 15–19]. IR, high proinflammatory cytokine levels, hormonal changes due to aging, decreased physical activity, and oxidative stress (Os) all promote SO and are common in the pathophysiology of obesity and sarcopenia. Among these factors, Os is a critical factor in the development of aging and obesity and, therefore, strongly influences SO. This review is aimed at describing SO as a highly prevalent condition and examining the role of Os in its pathophysiology.

2. Sarcopenic Obesity: General Characteristics

The prevalence of SO varies between 2% and 85%. This wide range depends on the heterogeneity of SO definitions, the analyzed population, and the different criteria and/or diagnostic methods of obesity and sarcopenia [7, 19–21].

For SO diagnosis, it is essential to consider sarcopenia and obesity. As such, it is difficult to reach a consensus due to the multiple methods of evaluation for each condition, the use of some imprecise techniques (such as body mass index (BMI)), and the existence of different cut-off points for some values according to the population to be evaluated [12] (see Table 1). Actually, the SO diagnosis achieves through an assessment of skeletal muscle mass measured by computed tomography (CT) at the L3 level corrected for height squared (named skeletal muscle index) and BMI (>25 or 30 kg/m^2). However, there are no internationally standardized criteria for diagnosing SO [22]. Other diagnostic methods include dual X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), and bioimpedance analysis (BIA). Still, all these methods are complex and costly and less frequently used in clinical practice. These methodologies are also a challenge to perform large-scale research and compare data between studies [19]. For these reasons, it is crucial to identify feasible methods for clinical use that allow a precise diagnosis of SO.

Since SO is composed of sarcopenia and obesity, we shall provide details of the relevant aspects of both conditions.

2.1. Sarcopenia. Sarcopenia is defined as a "syndrome with progressive and generalized loss of skeletal muscle mass, strength and physical function, which in turn is associated with an increased risk of adverse outcomes, such as physical disability, poor quality of life and higher mortality" [23–25]. It is classified as primary (associated with aging) or secondary (associated with limited mobility, malnutrition, or chronic diseases, such as obesity) [26, 27]. According to the European Working Group on Sarcopenia in Older People (EWGSOP), the diagnosis of sarcopenia is based on the presence of three criteria: (i) loss of muscle strength (a leading indicator of sarcopenia), (ii) decrease in the quantity or quality of muscle mass, and (iii) low physical performance [23, 26, 28]. The sarcopenia diagnosis is challenging due to the different tests and commonly used tools. In Table 1, we describe the primary diagnostic forms of sarcopenia in both clinical and research settings.

Table 1: Diagnosis of sarcopenia and obesity.

Sarcopenia diagnosis	
Muscle mass	
Clinical setting	Extremity circumferences (thigh, arm) Anthropometry Total or partial body potassium per fat-free soft tissue MAMA (middle-arm muscle area)
Research setting	DEXA (dual-energy X-ray absorptiometry) Thigh US (ultrasound) BIA (bioelectrical impedance analysis) Magnetic resonance imaging (MRI)
Muscle strength	
Clinical setting	Handgrip strength Knee flexion/extension 1 maximum repetition (1RM) 10 maximum repetitions (10RM) Peak expiratory flow (specific to respiratory)
Research setting	Isokinetic evaluation Dynamometer
Physical performance	
Clinical setting	Gait speed Short physical performance battery 6-MWT (6-minute walk test) 2-MST (2-minute step test) Chair stands Timed get-up-and-go test Stair climb power test
Research setting	CPET (cardiopulmonary exercise testing)
Obesity diagnosis	
Clinical setting	Body mass index (BMI) (≥30 kg/m^2) Fat mass (FM) % (>25% for men and >35% for women) Waist circumference (≥88 cm for women and 102 cm for men) Waist-to-hip ratio (WHR) Waist-to-height ratio (WHTR) Extremity circumferences (thigh, arm)
Research setting	DEXA (dual-energy X-ray absorptiometry) US (ultrasound) BIA (bioelectrical impedance analysis)

[26, 28, 40, 148, 157–159].

Sarcopenia is clinically relevant because the World Health Organization (WHO) has recognized it as a disease and included it in the International Classification of Diseases (ICD code M62.8) [29]. Furthermore, it is a critical determinant of frailty that leads to loss of autonomy and functionality in daily activities. Besides, sarcopenia increases hospitalization, osteoarthritis, osteoporosis, and the risk of institutionalization [30].

2.2. Obesity. The WHO defines obesity as "abnormal or excessive fat accumulation that may impair health" and an obese person as someone with a body mass index (BMI) greater than or equal to 30 [31, 32]. The WHO recognizes obesity as a chronic and progressive disease with a high chance of relapse, so it is considered a world epidemic [33]. The obesity diagnosis can be achieved in clinical settings through BMI, waist circumference, waist-to-hip ratio (WHR), waist-to-height ratio (WHTR), and fat mass. In the research context, obesity is usually diagnosed using DEXA, US, and BIA (see Table 1) [34–36].

The obesity diagnosis is marked by difficulties, particularly in relation to BMI. Although BMI is widely used around the world to diagnose obesity, it is an imprecise method because it does not discern between lean and fat mass, neither does it specify fat quantity or distribution [37]. Also, ethnic differences in BMI values have been observed (e.g., Asian population) [38, 39]. Furthermore, BMI is not the best method to determine obesity in the elderly because there are changes in the body composition during aging (body fat redistribution and muscle mass and bone density reductions), affecting the cut-off points for BMI [8, 34, 40].

Obesity is a risk factor in developing other diseases such as cardiovascular diseases (atherosclerosis, myocardial infarct, heart failure, and coronary disease), metabolic syndrome, T2DM, NAFLD, cirrhosis, cancer, osteoarthritis, pulmonary dysfunction (e.g., obstructive sleep apnea syndrome), reduced cognitive skills, urinary incontinence, and, more recently, coronavirus disease 2019 (COVID-19) [41–48].

3. Pathophysiology of Sarcopenic Obesity

Obesity and sarcopenia have common pathological features that could promote their development, such as IR, increased proinflammatory cytokines, age-associated hormonal changes, decreased physical activity, and Os, as well as liver, adipose, and skeletal muscle dysfunction. In this review, we focus on establishing how these factors affect skeletal muscle to generate sarcopenia. We also emphasize the role of Os in the pathophysiology of SO (Figure 1).

3.1. Insulin Resistance. IR is a feature of aging and obesity in humans and rodents. Aging could increase body fat mass, mainly in the abdominal area (visceral fat), which is most common in women than in men—this is called abdominal obesity. Furthermore, in aging, increased intramuscular (myosteatosis) and intrahepatic (liver steatosis) fat deposits induce IR [31, 32]. Interestingly, the decrease in elevated insulin levels and reduction in fat percentage could reverse obesity in older people [19, 31, 33].

Pathological myosteatosis in aging and obesity is associated with decreased insulin sensitivity and muscle mass and strength loss. The mechanism involves the impaired insulin signaling by interacting with lipidic intermediates such as diacylglycerol (DAG), long-chain acyl-coenzyme A, and ceramide. These interactions at various levels inhibit the GLUT-4 translocation to the sarcolemma. Together with these events, the decreased insulin secretion by the pancreas is derived from elevated concentrations of fatty acids, which induces β-cell apoptosis and reduces proliferation of pancreatic cells [14, 16, 34–36].

3.2. Inflammatory State: Chronic Systemic Inflammation. Systemic chronic inflammation is the primary factor influencing SO pathophysiology. Thus, the chronic inflammatory state in obesity and aging has harmful effects on skeletal muscle, inhibiting protein synthesis, decreasing oxidative capacity, and developing IR.

In obesity, the activation of macrophages, inflammatory T lymphocytes, and mast cells results from higher fat mass and adipocyte hypertrophy, creating a low proinflammatory state and an imbalance of adipokines. The characteristic profile of soluble factors in obesity and aging, such as decreased adiponectin, elevated levels of C-reactive protein (CRP), leptin, tumor necrosis factor-α (TNF-α), and interleukin 6 (IL-6), could lead to progressive loss of muscle mass and an increase in fat mass [14, 16, 37–40].

CRP is a marker of systemic inflammation. It increases in the elderly and is related to sarcopenia and SO [41, 42]. High leptin levels in aging and obesity upregulate the proinflammatory cytokines IL-6 and TNF-α, reducing insulin-like growth factor 1 (IGF1) activity and decreasing their anabolic actions on skeletal muscle [14, 43, 44]. TNF-α is a proinflammatory cytokine that increases in aging and obesity, promotes protein degradation, decreases protein synthesis, and inhibits myogenic differentiation [14, 45]. Also, adiponectin and growth hormone (GH) decrease their secretion in obesity and aging, inducing adverse effects on muscle protein synthesis. This effect could be associated with higher levels of "geriatric cytokines," such as IL-6 and CRP, which decrease muscle mass and strength [14, 38]. IL-6 is a myokine associated with sarcopenia and is upregulated in older persons [31, 38, 41, 44, 46–48]. Furthermore, aging-induced myosteatosis promotes lipotoxicity (Lptx) and contributes to inflammation [49, 50].

3.3. Hormonal Changes. Aging comes with is a decrease in anabolic hormones such as testosterone and GH. In males, the testosterone level (including its precursor dehydroepiandrosterone sulfate) declines in aging up to 1% per year from 30. In women, testosterone also rapidly decreases from 20 to 45 years old. This effect could harm muscle protein synthesis. In obese people, testosterone levels are low [31, 41, 51–53].

GH circulant levels also decrease after 30 years of age at a rate of ~1% per year. These conditions induce loss of muscle mass and accumulation of visceral fat in the elderly [31, 54–58]. Significantly, high levels of circulating free fatty acids (FFA) in elderly obesity inhibit GH production and decrease plasma levels of IGF-I, associated with low muscle mass.

In menopausal women, low estrogen levels promote muscle mass decrease and fat mass increase, mainly in the abdominal area. The fat mass percentage increases waist circumference and cardiovascular risk. These effects could be mitigated with hormone replacement therapy [37, 59, 60].

Myostatin expression increases in skeletal muscle due to obesity and IR. Thus, it could favor the loss of skeletal muscle in SO [14, 61, 62].

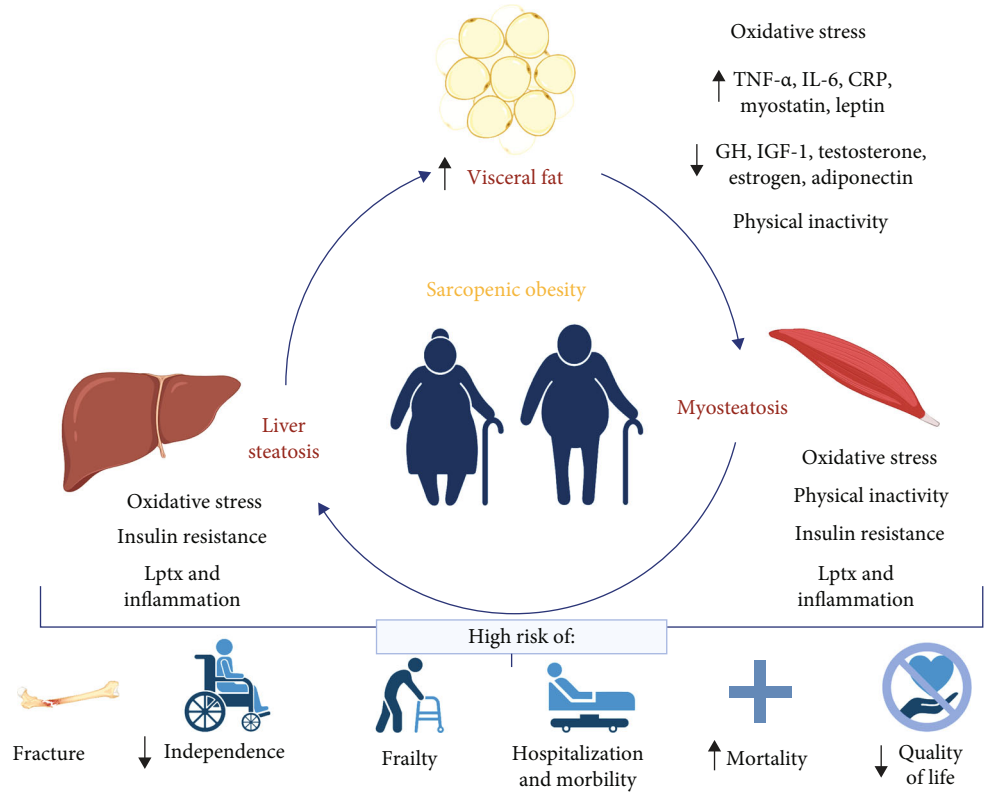

Figure 1: Pathophysiology and consequences of sarcopenic obesity. Sarcopenic obesity (SO) is a combination of obesity and sarcopenia in older people. Obesity and sarcopenia share pathological alterations such as insulin resistance, increased proinflammatory cytokines, age-associated hormonal changes, decreased physical activity, oxidative stress, and liver, adipose, and skeletal muscle dysfunction. Increased body fat mass, especially in the abdominal area (visceral fat), is characteristic of obesity and aging and produces an accumulation of adipose tissue in the liver (liver steatosis) and skeletal muscle (myosteatosis), with the consequent induction of IR, lipotoxicity (Lptx), inflammation, and oxidative stress (Os). Adipocyte hypertrophy induces a state of chronic systemic inflammation characterized by decreased adiponectin and elevated levels of C-reactive protein (CRP), leptin, tumor necrosis factor-α (TNF-α), and interleukin 6 (IL-6). Also, obesity and aging produce hormonal changes such as a decrease in growth hormone (GH), testosterone, estrogen, IGF-1, and adiponectin and an increase in myostatin. Finally, physical inactivity is a common feature of obesity and aging, affecting respiratory, osteoarticular, and neuromuscular levels, inducing loss of physical function. The consequences of sarcopenic obesity are a high risk of fractures, frailty, hospitalization, morbidity and mortality, loss of independence, and decreased quality of life. Abbreviations: SO: sarcopenic obesity; Lptx: lipotoxicity; Os: oxidative stress; CRP: C-reactive protein; TNF-α: tumor necrosis factor-α; IL-6: interleukin 6; GH: growth hormone; IGF-1: insulin-like growth factor 1.

3.4. Decrease of Physical Activity. The increase in adipose tissue in obesity can interfere with physical activity, leading to lower energy expenditure, favoring an increase in adipose tissue, and producing a vicious circle. Pathophysiological changes in the respiratory system, such as reduced lung and chest wall compliance caused by excess visceral fat, diminish the expiratory reserve volume (ERV) and functional residual capacity (FRC), increase pleural pressure, and cause ventilation and perfusion (V/Q) imbalance [63–65].

As mentioned earlier, physical inactivity and obesity increase the level of lipid circulation and myosteatosis in skeletal muscle, contributing to a decrease in muscle mass and strength and favoring sarcopenia and physical disabilities [14, 22, 66].

Furthermore, obesity in the elderly can favor joint dysfunction, chronic pain, disabilities relating to activities of daily living, and frailty, damaging functional status more than obesity or sarcopenia alone [31, 67–70].

Regarding aging, the limitation of physical activity can occur due to musculoskeletal disorders associated with advanced age, such as joint pain and stiffness. Sarcopenia can also induce the loss of physical function, leading to decreased physical activity and, therefore, an increase in adipose tissue and an augmented risk of obesity [19, 37]. As mentioned above, myosteatosis has been associated with aging, limiting functional activities due to decreased muscle mass and strength [71]. Muscle fibrosis is another pathological condition in aging. It is characterized by replacing skeletal muscle with fibrous connective tissue and impaired regenerative muscle capacity, decreasing muscle mass and functionality [71, 72].

Also, in aging, there are decreased rest metabolic rates (4% per decade after the age of 50 years), reduced motor neurons, and skeletal muscle metabolic adaptations, which could also favor obesity and loss of muscle mass [14, 31, 37, 73, 74].

3.5. Oxidative Stress. Oxidative stress (Os) is an imbalance of oxidant species and antioxidant systems towards an oxidative status, which is characterized by the accumulation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and cellular damage [75–77]. There are endogenous and exogenous sources of ROS and RNS. Endogenous sources include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), lipoxygenase, mitochondria, and xanthine oxidase. In contrast, exogenous sources include air and water pollution, tobacco, alcohol, heavy metals, drugs, industrial solvents, cooking pollutants, and radiation [75, 78, 79].

Antioxidants are the defense system against ROS-induced toxicity. Endogenous antioxidants include enzymatic, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and nonenzymatic, such as bilirubin and β-carotene. Exogenous antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), and phenolic antioxidants (such as resveratrol, phenolic acids, flavonoids, selenium, zinc, and acetylcysteine) [75, 80, 81].

Under normal conditions, ROS and RNS play a vital role in metabolism, immune response, and cellular proliferation and differentiation. In pathological conditions, there is increased production of ROS and RNS, together with insufficient antioxidant capacity. Os develops under these conditions, causing damage in organelles, carbohydrates, proteins, nucleic acids, and lipids, favoring their dysfunction [76–78].

4. Oxidative Stress in Sarcopenic Obesity

The oxidation-inflammatory theory of aging or "oxi-inflamm-aging" proposes that, during aging, chronic Os impairs the immune system, induces an inflammatory state, and creates a vicious circle of Os-inflammation-Os that damages structures, tissues, and organs [75, 82]. In obesity, high Os is associated with Lptx, inflammation, and IR in the liver, skeletal muscle, and adipose tissue [40, 83].

Sarcopenia and obesity are associated with Os through mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and imbalance of the muscle mass control pathways. These alterations are detailed below (Figure 2).

4.1. Mitochondrial Dysfunction. Os in sarcopenia induces mitochondrial dysfunction due to mitochondrial deoxyribonucleic acid (DNA) damage and impaired mechanisms for repairing DNA by excessive ROS. Moreover, muscle abilities for removing dysfunctional mitochondria become deficient, perpetuating Os [75, 84, 85]. Consequently, there is a decrease in mitochondrial quantity and quality, impairing the capacity to generate adenosine triphosphate (ATP), activating apoptotic pathways, and inducing the loss of muscle fibers [45, 75, 84, 86]. In this regard, aging causes the loss of type II muscle fibers more than type I, probably because type II fibers have a low mitochondrial quantity, making them more susceptible to degradation and loss of muscle mass [75, 87, 88].

In obesity, Os also inhibits mitochondrial function, resulting in Lptx, which impairs insulin signaling (a powerful anabolic signal), promotes high catabolism (which induces muscle mass loss), and leads to IR and inflammation [40, 89].

4.2. Endoplasmic Reticulum (ER) Stress. ER stress is induced by ROS accumulation, promoting Os. Obesity, metabolic syndrome, and aging cause ER stress and Os [90–93]. The ER stress and Os in these conditions are related to the "nutrient-sensing" functions of ER, which affect metabolic response at the endocrine and systemic levels [91]. Excess nutrients (ingesting high fat and/or high glucose), chronic inflammatory state, high adiposity, IR, metabolic syndrome, and aging harm the ER function in the liver, skeletal muscle, and adipose tissue, inducing Os [83, 91–95].

In the early stages of metabolic dysregulation, insulin secretion is elevated to compensate for increased glycemia (hyperinsulinemia). The high amount of insulin produced by the pancreas requires that the ER guarantee the correct folding of the hormone, which generates an ER overload and dysfunction, an unfolded protein response (UPR) overactivation, Os, and inflammation [83, 91–95]. Also, with aging and obesity (especially abdominal obesity), insulin sensitivity gradually decreases in skeletal muscle and adipocytes, increasing serum glucose levels and promoting ER overload and Os [83, 91–95].

In the liver, the imbalance in insulin metabolism negatively affects protein synthesis, lipogenesis, lipid transportation, and gluconeogenesis, inducing ER dysfunction and, consequently, Os. Also, adipocytes from obese and insulin-resistant subjects (humans and mice) present elevated lipid storage, lipogenesis, and adipokine synthesis, all of which induce ER stress and Os [83, 91, 96].

ER stress and Os develop during aging due to protein aggregation, damaged or misfolded proteins, an impaired protein cleansing system (by declining autophagic and proteasomal degradation), imbalance in calcium homeostasis, and decreased global protein synthesis. These conditions contribute to decreased skeletal muscle mass in aging [93, 97–101]. Furthermore, physical inactivity, a feature typically observed in aging and obesity, favors ER stress and UPR overactivation, inducing Os [93, 102].

4.3. Imbalance in Muscle Mass Control. Skeletal muscles are damaged by Os caused by ROS/RNS accumulation (mainly superoxide anions and hydrogen or peroxyl radicals) and a decrease in antioxidant activity (lower activities of SOD and CAT enzymes). Os leads to an imbalance in protein metabolism, favoring the catabolic pathway and decreasing the anabolic pathway activity. Thus, Os could play a fundamental role in losing the muscle mass that characterizes SO and promote IR [76, 92, 103]. Next, we examine the effects of Os in the control pathways of muscle mass and its impact on satellite cells.

4.3.1. Anabolic Pathway. A critical anabolic way for protein is the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway. This pathway is stimulated by insulin, insulin-like growth factor 1 (IGF-1), exercise, and testosterone, all of which decrease with obesity and aging [104–106]. Protein synthesis

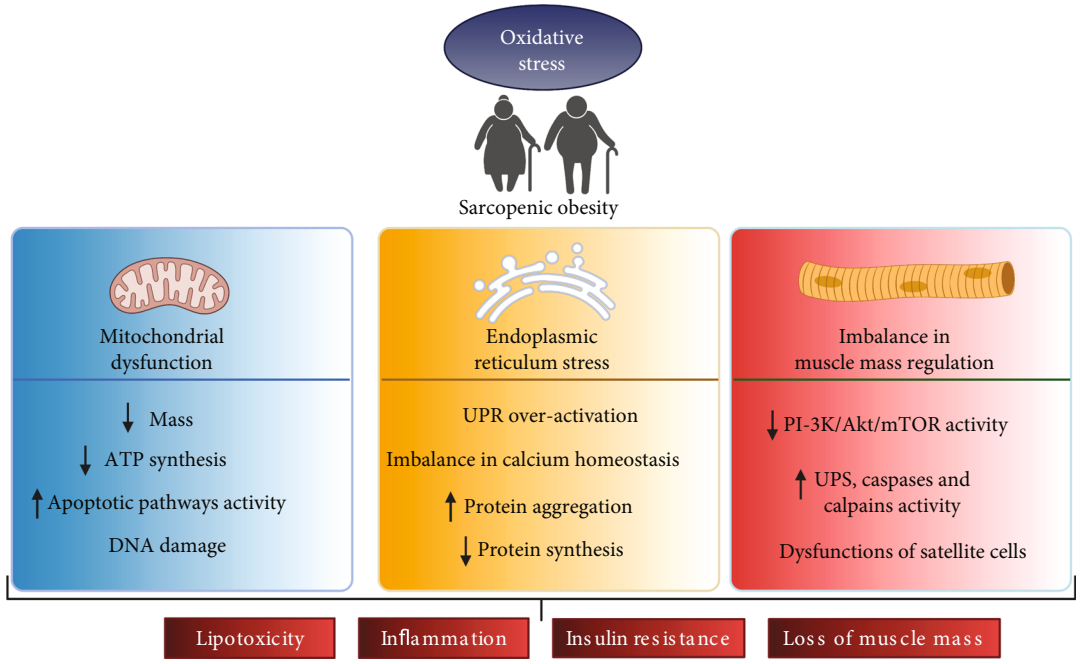

Figure 2: Oxidative stress in sarcopenic obesity. In older people, oxidative stress (Os) favors sarcopenia and obesity through mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and imbalance in muscle mass control. *Mitochondrial dysfunction* in sarcopenia is induced by Os due to mitochondrial DNA damage and impaired mechanisms for repairing DNA ability, impaired capacity to remove dysfunctional mitochondria, decreased mitochondrial quantity and quality, and impaired capacity to generate ATP to activate the apoptotic pathways. *ER stress* and Os are caused by an increase in adipose tissue, chronic inflammation, and insulin resistance, all of which are characteristics of obesity and aging. ER stress induces Os, favoring unfolded protein response (UPR) overactivation, imbalance in calcium homeostasis, increased protein aggregation, and decreased protein synthesis. *Imbalance in muscle mass control* occurs because Os increases the catabolic activity and decreases the anabolic pathway in muscle mass control. Os reduces protein synthesis due to the reduced activity in phosphatidylinositol 3-kinase (PI3K)/serine-threonine kinase (Akt)/mammalian target of rapamycin (mTOR). Os increases the activity of the ubiquitin-proteasome system (UPS) and activates muscle proteases such as caspases and calpains. Finally, due to Os, satellite cells' quantity and regenerative function decline with age and obesity. Mitochondrial dysfunction, ER stress, and imbalance in the muscle mass control pathways induce lipotoxicity (Lptx), chronic inflammation, IR, and loss of muscle mass, affecting physical function and independence in sarcopenic obesity. Abbreviations: Os: oxidative stress; ER: endoplasmic reticulum; UPR: unfolded protein response; PI3K: phosphatidylinositol 3-kinase; Akt: serine-threonine kinase; mTOR: mammalian target of rapamycin; UPS: ubiquitin-proteasome system (UPS); Lptx: lipotoxicity.

is reduced under Os conditions [107, 108], and Os promotes the activation of pathways such as c-Jun N-terminal kinase (JNK), IκB kinase (IKK), and p38 mitogen-activated protein kinase (p38-MAPK), leading to the inactivation of the insulin receptor. Indeed, they favor IR and low anabolic activity in skeletal muscle [40, 109]. In sarcopenia, the loss of muscle mass and strength leads to reductions in physical activity and mobility, inducing Os, exacerbating sarcopenia, and generating an endless circle [107, 108, 110].

Moreover, in physiological conditions, PI-3K/Akt inhibits the forkhead box transcription factor O (Fox-O) (a potent inductor of the ubiquitin-proteasome system (UPS)), while mTOR decreases caspase activity. PI-3K/Akt/mTOR activity declines during aging, promoting the catabolic pathway. Also, physical inactivity (a characteristic of obesity and sarcopenia in aging) indirectly inhibits the mTOR pathway through Fox-O stimulation [107, 108].

4.3.2. Catabolic Pathway. Elevated ROS levels activate the UPS, increasing expression of the muscle-specific ubiquitin ligase MuRF1 (Muscle RING-finger protein-1) and atrogin-1. ROS also activates muscle proteases such as caspases and calpains, leading to protein breakdown [87, 105, 108, 111, 112].

Another redox-sensitive transcription factor is nuclear factor kappa B (NF-κB), which dramatically increases muscle activity in sarcopenia, metabolic syndrome, and obesity [83, 113]. NF-κB regulates the expression of myokines such as TNF-α and IL-6, inducing chronic low-grade inflammation and apoptosis. These conditions increase catabolic pathway activity and decrease anabolic pathway activity in skeletal muscle, promoting the loss of muscle mass and strength [76, 114]. Furthermore, in obesity, NF-κB can be activated by different stimuli such as lipopolysaccharide, free fatty acids (FFAs), advanced glycation end products, inflammatory cytokines, Os, and ER stress. NF-κB activation induces inflammation and IR, favoring catabolic activity, and decreases anabolic pathways in skeletal muscle [76, 83].

4.3.3. Satellite Cells. The function of satellite cells in muscle regeneration and its decline with age contributes to lower capacities to self-renew and regenerate muscle tissue [87,

115, 116]. The reduction in these cells has been explicitly shown in type II fibers more than in type I during aging [87, 117]. Also, myosteatosis, typically displayed in aging, could impair muscle fiber, replace muscle tissue, decrease muscle protein synthesis, and impair new muscle tissue growth [48, 66, 118–120].

The increased ROS levels and decreased antioxidant activity in satellite cells [121, 122] could dysregulate basal autophagy (essential to maintaining the quiescent state of stem cells) and impair the removal of misfolded proteins, thereby affecting satellite cell homeostasis [87, 116]. In addition, Os present in the elderly dysregulates the typical functions and processes of satellite cells such as proliferation, fibrosis, and differentiation involving Notch, Wnt, p38/MAPK, and the JAK-STAT3 signaling pathways [72, 123–125].

Furthermore, satellite cells in obesity and overweight have a minor expression and activity of myogenic regulatory factors (MRFs) such as MyoD, Myf5, and Myf6. MRFs are regulators of the myogenic differentiation of satellite cells in various stages. The decreased activity of MRF is due to dysregulated autophagy concerning inflammation and IR, also leading to a reduction in satellite cells [92, 116, 126, 127].

In obesity, satellite cells acquire adipocyte features, expressing adipocyte-specific genes and accumulating lipids, with a likely effect that favors myosteatosis [128–130].

5. Redox-Dependent Mechanisms in Sarcopenic Obesity

There is limited evidence to clarify the mechanisms involved in the redox-dependent effect of SO in human and animal models. Below, we will present information related to the more probable mechanisms involved in Os effects in SO.

5.1. Animal Models. Most of the research that links Os and SO in animals is focused on testing agents with antiobesity, antiaging, or antioxidant effects. The results did not directly elucidate the mechanisms by which Os favors SO, but they help understand the associated events to this pathological condition.

Resveratrol, a natural and botanical polyphenol, administered to rats fed with a high-fat diet (HFD), prevented typical SO features such as muscle mass loss, myofiber size decrease, decreased muscle strength, and excessive muscle fat accumulation. The preventive mechanism involved the PKA/LKB1/AMPK pathway [131]. Tocotrienols (TT) and green tea polyphenols (GTP) are other antioxidant agents that increased muscle mass and cross-sectional area (CSA) and increased the mitochondrial enzyme activity in animal models of obesity [132].

Another therapeutical strategy has been the administration of probiotic *Lactobacillus paracasei* PS23 (LPPS23) to aged mice. The effect showed a deacceleration and attenuation of the decline in muscle mass and strength. Mechanistically, treatment with LPPS23 produced a higher mitochondrial function, antioxidant enzymes, and lower inflammatory cytokines and Os [133].

BAM15, a mitochondrially targeted protonophore with wide tolerability, stimulated energy expenditure and glucose and lipid metabolism to prevent diet-induced obesity in mice. Besides, BAM15 improved glycemic control and reduced adiposity through insulin signaling and oxidation of glucose and fatty acids in an AMPK-dependent manner [134].

BDA-410 is a synthetic calpain inhibitor that induced loss of weight and body fat mass in aged mice [135]. In skeletal muscle, BDA-410 improved the skeletal muscle contractility by mechanisms dependent on enhanced lipolysis and excitation-contraction coupling, favoring a lean phenotype [135].

Fucoxanthinol (FXOH) (a metabolite of fucoxanthin (FX) that has antiobesity effects and that accumulates in white adipocytes of mice) showed antisarcopenic and antiobesity activities *in vitro*, mainly by decreasing muscle atrophy, incrementing lipolysis, and decreasing triglyceride (TG) content. Interestingly, the effects of FXOH were dependent on Os [136].

Angiotensin 1-7 (Ang-(1-7)) is a small endogenous peptide that belongs to the renin-angiotensin system [137]. The administration of Ang-(1-7) to mice with obesity or metabolic syndrome reduced body weight, upregulated thermogenesis and brown adipose tissue (BAT), and ameliorated impaired glucose [138]. In obese rats, Ang-(1–7) enhanced glucose tolerance, insulin sensitivity, and decreased plasma insulin levels, as well as a significant decrease in circulating lipid levels [139]. In obese humans, Ang-(1-7) administration decreased vascular dysfunction related to impaired insulin sensitivity [140]. Regarding skeletal muscle, there is broad evidence about the antiatrophic and antisarcopenic role of Ang-(1-7) [141–144]. Despite the fact that the effect of Ang-(1-7) has not been directly assayed in SO, the mechanisms involved in muscle mass regulation include decreased protein degradation, prevention of Os, apoptosis, and mitochondrial dysfunction. These features are separately present in skeletal muscle from obese and aged mice [145–147].

5.2. Patients. There is little evidence of the Os-dependent effect in SO patients that could guide the mechanisms involved. Circulating markers of Os (such as GSH, oxidized glutathione (GSSG), plasma malondialdehyde (MDA), and 4-hydroxy 2-nonenal (4-HNE)) increase in elderly patients with SO compared to nonsarcopenic nonobese, sarcopenic nonobese, and nonsarcopenic obese patients. These findings confirm that Os is related to SO [148]. Also, systemic Os is associated with a decline in muscle mass in elderly patients with obesity and T2DM [149]. Furthermore, a study identified biomarkers of inflammation and Os, such as serum adiponectin, the erythrocyte sedimentation rate (ESR), and CRP levels, as being associated with sarcopenia [150]. Interestingly, a recent study showed that a moderate-intensity exercise program reduces oxidative damage and increases the antioxidant system, thereby serving as a feasible tool for treating SO [151].

6. Perspectives in Sarcopenic Obesity

Research on the effects of Os on SO is undoubtedly necessary to understand the influence and mechanisms involved in controlling metabolism and muscle mass. It is also essential to harmonize the criteria that define and diagnose

sarcopenia and obesity. Similarly, there is a need for advances in reaching a consensus in the methodology for the SO diagnosis that can be applicable in different populations, ideally used in clinical practice, and feasible for use in long-standing studies [3, 7, 12, 16, 37, 48, 152, 153]. Further, since SO is a multifactorial disease, the treatment must also be multifactorial [31]. The treatment could include exercise training and nutritional, pharmacological, psychological, and social interventions [5, 6, 12, 48, 154–156]. One problem is the elevated cost of a multifactorial intervention, which health insurance generally does not cover. Considering these antecedents, prevention could be fundamental. Ideally, the prevention of SO should start early in life, continuing in later stages [31].

7. Conclusions

SO is a highly prevalent condition that includes obesity and sarcopenia in aging, which are also highly prevalent. SO increases the risk of physical functional decline in older adults, favoring high morbimortality in patients. The SO diagnosis is the primary difficulty to overcome. There is no consensus on evaluation methods and definitions of SO. Therefore, results from different investigations are highly variable and, thus, difficult to compare.

The pathophysiological factors influencing SO are Os, IR, chronic low-grade inflammation, age-associated hormonal changes, and decreased physical activity. Os is a condition that affects the three main organs and tissues involved in SO (the liver, adipose tissue, and skeletal muscle), leading to a vicious cycle of oxidative damage and inflammation that induces tissue dysfunction. Os-dependent damage due to SO includes mitochondrial dysfunctions and ER stress, which affect the liver, adipose tissue, and skeletal muscle. Also, there is an imbalance in the control muscle mass pathway and satellite cell function that directly affects muscle mass.

Abbreviations

Akt: Protein kinase B
AMPK: AMP-activated protein kinase
ATP: Adenosine triphosphate
BAM15: ((2-Fluorophenyl) {6-[(2-fluorophenyl) amino] (1,2,5-oxadiazolo[3,4-e] pyrazin-5-yl)} amine)
BIA: Bioimpedance analysis
BMI: Body mass index
CAT: Catalase
CRP: C-reactive protein
CT: Computed tomography
DAG: Diacylglycerol
DNA: Deoxyribonucleic acid
DXA: Dual X-ray absorptiometry
ER: Endoplasmic reticulum
ERV: Expiratory reserve volume
ESR: Erythrocyte sedimentation rate
EWGSOP: European Working Group on Sarcopenia in Older People
FFAs: Free fatty acids
FRC: Functional residual capacity
FX: Fucoxanthin
4-HNE: 4-Hydroxy 2-nonenal
Fox-O: Forkhead box transcription factor O
FXOH: Fucoxanthinol
GH: Growth hormone
GSH: Reduced glutathione
GSH-Px: Glutathione peroxidase
GSSG: Oxidized glutathione
HFD: High-fat diet
IGF-1: Insulin-like growth factor 1
IKK: IκB kinase
IL-6: Interleukin 6
IR: Insulin resistance
JAK-STAT3: Janus kinase-signal transducer and activator of transcription proteins
JNK: c-Jun N-terminal kinase
LPPS23: Probiotic Lactobacillus paracasei PS23
LKB1: Liver kinase B1
Lptx: Lipotoxicity
MDA: Malondialdehyde
MFRs: Myogenic regulatory factors
MPO: Myeloperoxidase
MRI: Magnetic resonance imaging
mTOR: Mammalian target of rapamycin
MuRF1: Muscle RING-finger protein-1
NADPH: Nicotinamide adenine dinucleotide phosphate
NAFLD: Nonalcoholic fatty liver diseases
NF-Kb: Nuclear factor kappa B
Os: Oxidative stress
PA: Palmitate acid
PI: Physical inactivity
PI3K: Phosphatidylinositol 3-kinase
PKA: Protein kinase A
p38-MAPK: p38 mitogen-activated protein kinase
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SO: Sarcopenic obesity
SOD: Superoxide dismutase
TG: Triglyceride
TNF-α: Tumor necrosis factor-α
T2DM: Type 2 diabetes mellitus
UPR: Unfolded protein response
UPS: Ubiquitin-proteasome system
US: Ultrasound
V/Q: Ventilation and perfusion
WHO: World Health Organization
WHR: Waist-to-hip ratio
WHTR: Waist-to-height ratio.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Research Article

Salivary Redox Biomarkers in Insulin Resistance: Preclinical Studies in an Animal Model

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Insulin resistance (IR) is a condition of impaired tissue response to insulin. Although there are many methods to diagnose IR, new biomarkers are still being sought for early and noninvasive diagnosis of the disease. Of particular interest in laboratory diagnostics is saliva collected in a stress-free, noninvasive, and straightforward manner. The purpose of the study was to evaluate the diagnostic utility of salivary redox biomarkers in preclinical studies in an animal model. The study was conducted on 20 male Wistar rats divided into two equal groups: a standard diet and a high-fat diet (HFD). In all rats fed the HFD, IR was confirmed by an elevated homeostasis model assessment (HOMA-IR) index. We have shown that IR is responsible for the depletion of the enzymatic (↓superoxide dismutase) and nonenzymatic (↓ascorbic acid, ↓reduced glutathione (GSH)) antioxidant barrier at both the central (serum/plasma) and salivary gland (saliva) levels. In IR rats, we also demonstrated significantly higher concentrations of protein/lipid oxidation (↑protein carbonyls, ↑4-hydroxynoneal (4-HNE)), glycation (↑advanced glycation end products), and nitration (↑3-nitrotyrosine) products in both saliva and blood plasma. Salivary nonenzymatic antioxidants and oxidative stress products generally correlate with their blood levels, while GSH and 4-HNE have the highest correlation coefficient. Salivary GSH and 4-HNE correlate with body weight and BMI and indices of carbohydrate metabolism (glucose, insulin, HOMA-IR) and proinflammatory adipokines (leptin, resistin, TNF-α). These biomarkers differentiate IR from healthy controls with very high sensitivity (100%) and specificity (100%). The high diagnostic utility of salivary GSH and 4-HNE is also confirmed by multivariate regression analysis. Summarizing, saliva can be used to assess the systemic antioxidant status and the intensity of systemic oxidative stress. Salivary GSH and 4-HNE may be potential biomarkers of IR progression. There is a need for human clinical trials to evaluate the diagnostic utility of salivary redox biomarkers in IR conditions.

1. Introduction

One of the most significant medical problems of the 21st century is the increased incidence of type 2 diabetes mellitus (DM2). Nowadays, more than 422 million people worldwide have diabetes, of which about 30-40% are still undiagnosed [1]. Several epidemiological studies have shown that DM2 complications cause disability and reduced quality of life for patients with diabetes [1, 2]. DM2 not only leads to micro- and macrovascular angiopathies but is also a significant cause of premature mortality in developed countries [3]. A key role in DM2 development has been attributed to

insulin resistance (IR), that is, decreased sensitivity of target tissues to insulin action [4]. IR enhances vascular endothelial proliferation, inflammation, and atherosclerotic plaque formation, mainly due to inhibition of the 3-phosphatidylinositol 3-kinase (PI-3K) and mitogen-activated protein kinase (MAP-kinases) pathways. In addition, IR is inextricably linked to obesity [4, 5]. Increased levels of free fatty acids (FFAs), adipokines, and proinflammatory cytokines inhibit insulin action by affecting the insulin receptor substrate (IRS-1) or impairing translocation of the glucose transporter GLUT4 [4–6]. Nevertheless, the common denominator of metabolic disorders in obesity, IR, and DM2 is also oxidative stress. Increased formation of reactive oxygen species (ROS) occurs not only under ceramide, diacylglycerol (DAG), and triacylglycerol (TAG) accumulation but also impaired insulin signaling and inflammation [7–9]. Oxidative stress has been shown to increase the expression of stress-activated kinases such as protein kinase C (PKC) and c-Jun N-terminal kinase (JNK), which blocks phosphorylation of IRS-1 tyrosine residues and thus leads to increased blood glucose levels [6, 9, 10]. Overproduction of ROS also results in the phosphorylation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) inhibitor, responsible for activating this transcription factor and inducing inflammation [6, 11]. It also enhances the synthesis of ceramide and, through positive feedback, increases the production of free radicals [12–14]. Therefore, oxidative stress is considered one of the most important pathological factors in the progression of metabolic diseases [6, 9, 10, 15, 16]. Therefore, it is not surprising that redox biomarkers have been postulated for the diagnosis of IR [17–22].

A biomarker is an objectively measurable change in a biological material, which may indicate a normal physiological state (clinical diagnosis), a pathological state (monitoring of disease progression), or a response to pharmacological treatment (evaluating the effectiveness of drug therapy) [23]. However, despite the tremendous development of diabetology and endocrinology, the current state of knowledge is still not sufficient to rapidly diagnose and treat the metabolic complications of IR. Therefore, alternative and effective diagnostic/treatment methods are constantly being sought [1]. Recently, saliva is of increasing interest in clinical diagnostics. Its advantages include low cost, high durability, and noninvasive and painless collection, including from children and disabled persons [24–27]. Although the main component of saliva is water, it also contains electrolytes, amino acids, proteins, lipids, hormones, vitamins, antioxidants, and oxidation products of biomolecules [28]. These compounds can pass from the blood to saliva by passive (simple diffusion, ultrafiltration, and facilitated diffusion) and active transport as well as through damaged cell membranes [29, 30]. Thus, saliva contains most of the compounds present in the plasma, which is the basis for its use in medical diagnostics. Despite many studies on the role of oxidative stress in the pathogenesis of IR [6, 9, 10, 15, 16], there are no data on the usefulness of salivary redox biomarkers in the diagnosis of insulin resistance. Therefore, our study is the first to evaluate the clinical utility of salivary antioxidants and oxidative stress products in a preclinical study in an animal model. In insulin-resistant rats, the activity/concentration of salivary redox biomarkers was compared with their plasma and serum levels and the classical biomarkers of metabolic disturbances.

2. Results

2.1. Animal Characteristics. The study was conducted on 20 male Wistar rats divided into two equal groups: a standard diet and a high-fat diet (HFD). Body weight, BMI, and energy intake were significantly higher in the HFD group than in the control group. Interestingly, the food intake in rats fed HFD was notably lower compared to the normally fed rats. Moreover, plasma glucose, insulin, leptin, and resistin concentrations were significantly higher in the HFD group compared to the control. HOMA-IR index was significantly higher in all HFD-fed animals confirming systemic IR (Table 1).

2.2. Enzymatic Antioxidants. Antioxidants are compounds that form a protective barrier in cells, neutralizing the effects of free radicals. Antioxidant enzymes include catalase (CAT), salivary peroxidase (Px)/glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD).

The analysis of enzymatic antioxidants revealed a statistically significant decrease in the activity of salivary Px ($\downarrow$36%, $p = 0.0029$, Figure 1(e)) and salivary SOD ($\downarrow$39%, $p \leq 0.0001$, Figure 1(m)), as well as serum CAT ($\downarrow$53%, $p \leq 0.0001$, Figure 1(b)) and serum SOD ($\downarrow$61%, $p \leq 0.0001$, Figure 1(n)) in the HFD group compared to the control. On the other hand, no notable difference in the activity of salivary CAT (Figure 1(a)), salivary GR (Figure 1(i)), serum GPx (Figure 1(f)), and serum GR (Figure 1(j)) between groups was observed.

Moreover, significantly higher saliva/serum ratio of CAT ($\uparrow$150%, $p \leq 0.0001$, Figure 1(c)) and SOD (64%, $p \leq 0.0012$, Figure 1(o)) in HFD rats was observed in comparison to the control group.

What is more, we observed a high positive correlation of SOD activity ($r = 0.7501$, $p = 0.0001$, Figure 1(p)) between serum and saliva in HFD rats. There were no notable correlations of the other investigated enzymatic biomarkers.

2.3. Nonenzymatic Antioxidant. Nonenzymatic antioxidants mainly include small molecular weight antioxidants such as ascorbic acid (AA), reduced glutathione (GSH), and uric acid (UA). Assays evaluating nonenzymatic antioxidant revealed that high-fat diet caused a significant decrease in the level of salivary ($\downarrow$52%, $p \leq 0.0001$, Figure 2(a)) and plasma AA ($\downarrow$19%, $p = 0.0014$, Figure 2(b)). Similarly, GSH concentration in the saliva ($\downarrow$58%, $p \leq 0.0001$, Figure 2(e)) and in the plasma ($\downarrow$61%, $p \leq 0.0001$, Figure 2(f)) in the HFD group was significantly lower than in the control group. What is more, a high-fat diet increased the concentration of UA in the saliva ($\uparrow$46%, $p = 0.001$, Figure 2(i)) and the plasma ($\uparrow$46%, $p = 0.0024$, Figure 2(j)) of HFD rats comparing to the control.

Table 1: General characteristics of the control and high-fat diet fed (HFD) rats.

Parameter	Control group	HFD group
Body weight (g)	388 ± 34.16	537 ± 36.52****
BMI (g/cm^2)	0.66 ± 0.024	0.90 ± 0.016****
Energy intake (g/rat/week)	206.4 ± 22.06	248.6 ± 38.23**
Food intake (g/day)	18.07 ± 1.93	11.05 ± 1.70****
Plasma glucose (mg/dL)	90.94 ± 3.31	146.4 ± 12.54****
Plasma insulin (mU/mL)	78.65 ± 7.55	172.30 ± 12.07****
HOMA-IR	1.72 ± 0.18	16.20 ± 0.65****
Plasma leptin (mU/mL)	26.84 ± 2.88	49.40 ± 6.24****
Plasma resistin (mU/mL)	184 ± 19.12	394 ± 39.51****
Plasma TNF-α (mU/mL)	1010 ± 115.2	3248 ± 588.5****

Abbreviations: BMI: body mass index; HFD: high-fat diet; HOMA-IR: homeostatic model assessment of insulin resistance; TNF-α: tumor necrosis factor α; **$p < 0.01$ vs. control; ****$p < 0.0001$ vs. control.

A significantly lower saliva/serum ratio of AA (↓39%, $p = 0.0006$, Figure 2(c)) in rats fed a high-fat diet was observed compared to the control group. Interestingly, there were no statistical differences in the saliva/plasma ratio of GSH (Figure 2(g)) and UA (Figure 2(k)).

Moreover, a highly positive correlation of GSH content ($r = 0.7993$, $p \leq 0.0001$, Figure 2(h)) in plasma and saliva of HFD rats was noticed. AA ($r = 0.5334$, $p = 0.0154$, Figure 2(d)) and UA ($r = 0.4943$, $p = 0.0267$, Figure 2(l)) levels were also positively correlated between rats' plasma and saliva.

2.4. Oxidative/Nitrosative Damage. Oxidation (carbonyl groups (PC); 4-hydroxynoneal (4-HNE)), glycation (advanced glycation end products (AGE) and nitration (3-nitrotyrosine (3-NT)) products of proteins and lipids are used to assess the damage caused by ROS and RNS. The oxidative and nitrosative stress markers revealed that high-fat diet increased concentrations of salivary, as well as plasma PC (↑71%, $p = 0.0018$, Figure 3(a); ↑46%, $p \leq 0.0001$, Figure 3(b), respectively), 3-NT (↑6%, $p = 0.0235$, Figure 3(e); ↑31%, $p = 0.0007$, Figure 3(f)), AGE (↑51% $p \leq 0.0001$, Figure 3(i); ↑92%, $p \leq 0.0001$, Figure 3(j)), and 4-HNE (↑292% $p \leq 0.0001$, Figure 3(m); ↑140%, $p \leq 0.0001$, Figure 3(n)).

A significantly lower saliva/serum ratio of 3-NT (↓21%, $p = 0.0182$, Figure 3(g)) in the HFD group compared to the control group was noticed. On the other hand, in HFD rats, a notably higher saliva/serum ratio of 4-HNE (↑66%, $p = 0.0008$, Figure 3(o)) was observed compared to the control group.

Interestingly, significant positive correlations of all of the oxidative and nitrosative damage products such as PC, 3-NT, AGE, and 4-HNE ($r = 0.4727$, $p = 0.0353$, Figure 3(d); $r = 0.4789$, $p = 0.0327$, Figure 3(h); $r = 0.4777$, $p = 0.0331$, Figure 3(l); $r = 0.8341$, $p \leq 0.0001$, Figure 3(p), respectively) were observed between plasma and saliva in the HFD group.

2.5. Correlations with Metabolic Parameters. The analysis of the metabolic parameters and investigated biomarkers revealed highly positive correlations between salivary CAT and salivary 4-HNE, as well as plasma glucose HOMA-IR, and BMI ($r = 0.693$, $p = 0.026$; $r = 0.651$, $p = 0.041$; $r = 0.712$, $p = 0.021$; $r = 0.715$, $p = 0.02$, respectively). Interestingly, the activity of salivary SOD was correlated only with plasma resistin ($r = -0.851$, $p = 0.002$). Moreover, the activity of salivary GSH correlated negatively with BW ($r = -0.667$, $p = 0.035$) and BMI ($r = -0.889$, $p = 0.001$), as well as plasma glucose ($r = -0.79$, $p = 0.006$), insulin ($r = -0.783$, $p = 0.007$), HOMA-IR ($r = -0.936$, $p = 0.00007$), and proinflammatory adipokines (leptin: $r = -0.757$, $p = 0.011$; resistin: $r = 0.685$, $p = 0.029$; TNF-α: $r = -0.639$, $p = 0.047$). What is more, we have shown highly positive correlation between the salivary UA concentration and plasma leptin, plasma glucose, BW, and BMI ($r = 0.92$, $p \leq 0.0001$; $r = 0.777$, $p = 0.008$; $r = 0.691$, $p = 0.027$; $r = 0.754$, $p = 0.012$, respectively). Furthermore, we observed the positive correlations of salivary 4-HNE and resistin ($r = 0.776$, $p = 0.008$). However, salivary 4-HNE correlated also with BW ($r = 0.605$, $p = 0.044$) and BMI ($r = 0.854$, $p = 0.002$), as well as indicators of carbohydrate metabolism-glucose ($r = 0.716$, $p = 0.02$) and HOMA-IR ($r = 0.592$, $p = 0.05$) (Figure 4).

2.6. Multiple Regression Analysis. Multiple regression analysis of salivary biomarkers showed that salivary GSH was negatively associated with HOMA-IR ($p = 0.0042$). Moreover, significant association between BMI and the activity of salivary GSH and concentration of salivary 4-HNE were observed. No significant associations between other salivary biomarkers and BMI or HOMA-IR were noticed (Table 2).

2.7. ROC Analysis of the Analyzed Redox Biomarkers. The ROC analysis revealed that most of the salivary redox biomarkers significantly differentiated rats fed a standard diet and a high-fat diet. The assessment of salivary Px, SOD, and GSH (sensitivity = 70%, specificity = 70%, $p = 0.0102$; sensitivity = 90%, specificity = 90%, $p = 0.0004$; sensitivity = 100%, specificity = 100%, $p = 0.0002$) clearly distinguished the HFD group from the control. Similarly, the salivary levels of AA, UA, PC, 3-NT, AGE, and 4-HNE (sensitivity = 90%, specificity = 90%, $p = 0.0002$; sensitivity = 80%, specificity = 80%, $p = 0.0025$; sensitivity = 80%, specificity = 80%, $p = 0.0032$; sensitivity = 70%, specificity = 70%, $p = 0.0343$; sensitivity = 90%, specificity = 90%, $p = 0.0009$; sensitivity = 100%, specificity = 100%, $p = 0.0002$) significantly differentiated the HFD rats from the normal diet group. Moreover, number of serum and plasma biomarkers, such as CAT, SOD, GSH, and 4-HNE ($p = 0.0002$), were characterized by a 100% of sensitivity and specificity in differentiating the HFD rats from the control rats (Table 3).

3. Discussion

This study is the first to evaluate the clinical utility of salivary redox biomarkers in IR. In preclinical studies in an animal model, we demonstrated that salivary GSH and 4-HNE

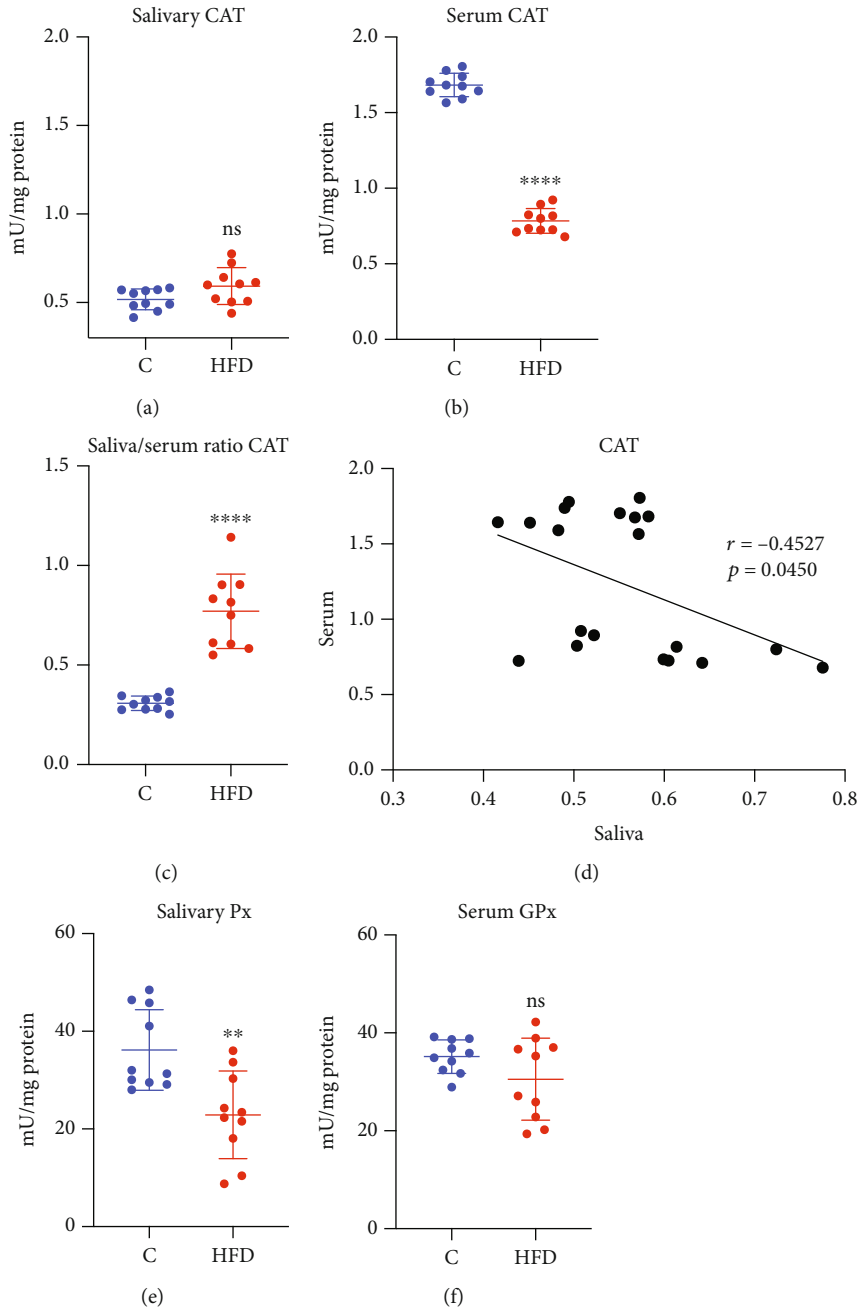
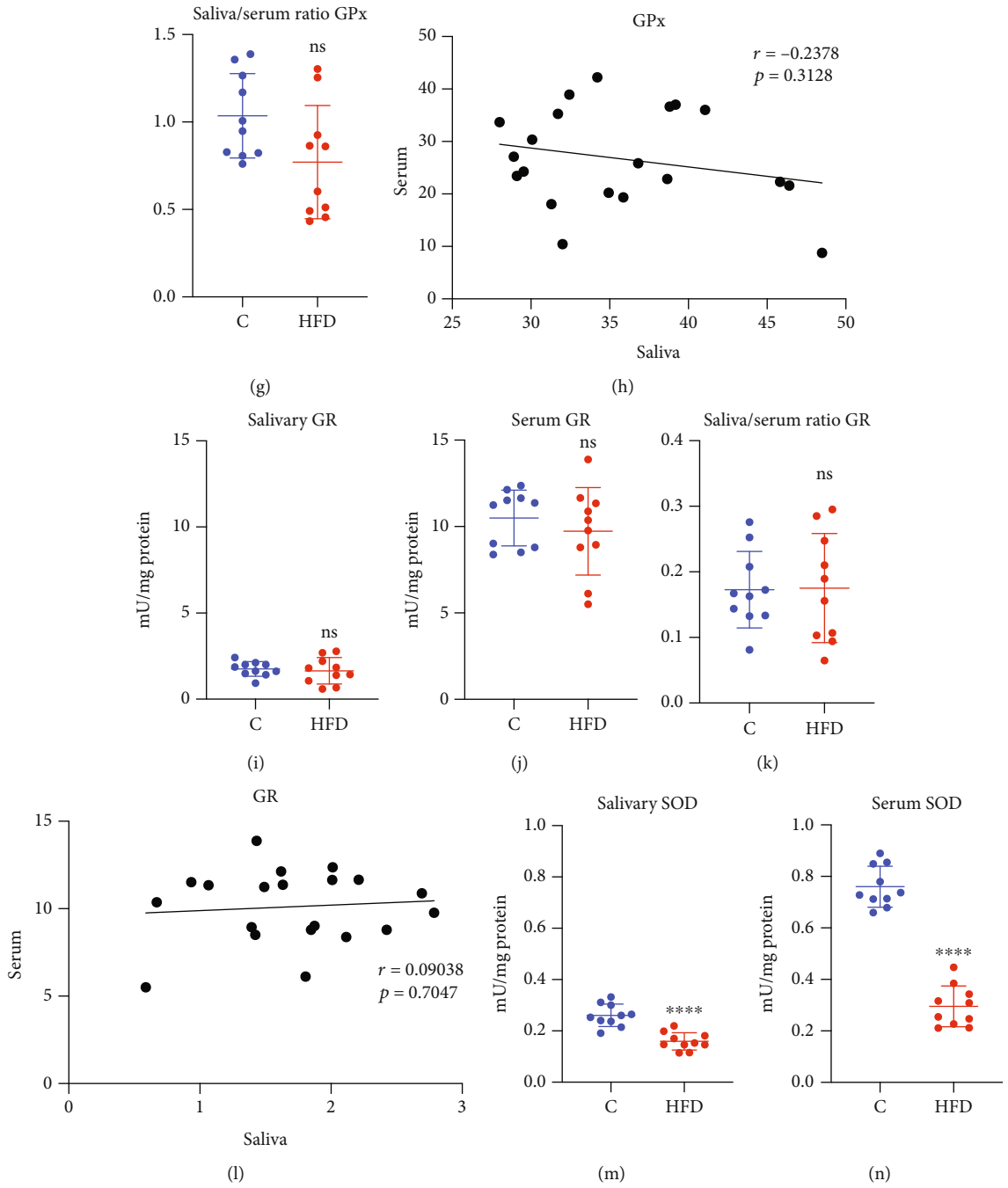


Figure 1: Continued.



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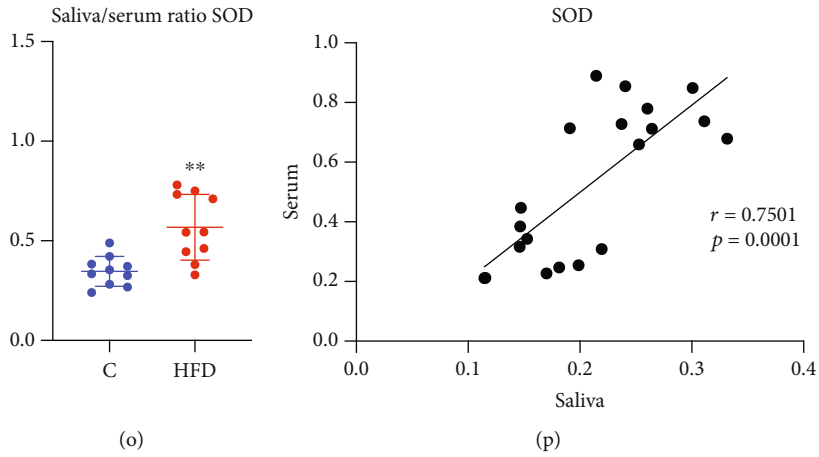
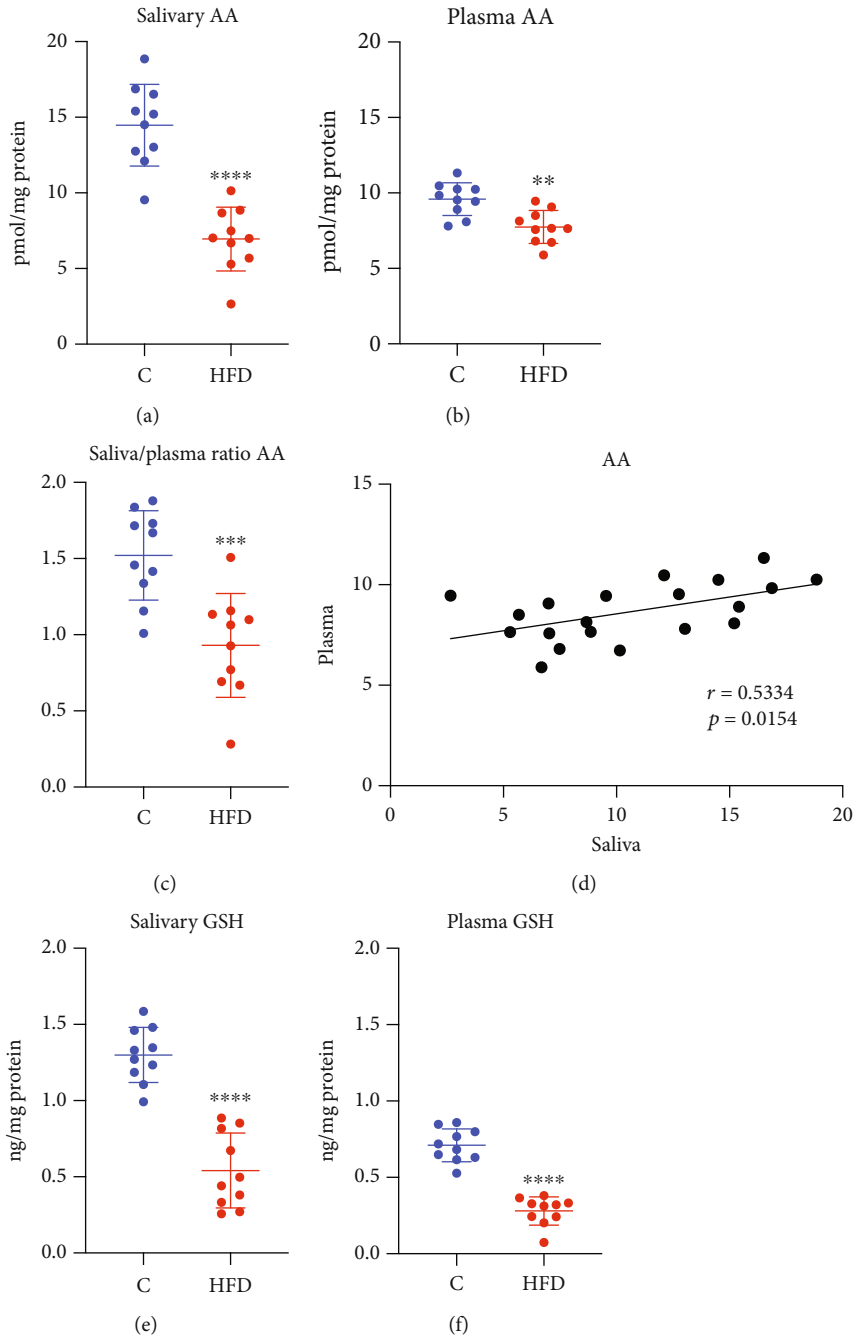


Figure 1: The effect of a high-fat diet (HFD) on salivary and plasma enzymatic antioxidants in rats. Abbreviations: C: control group; HFD: high-fat-diet group; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; Px: peroxidase; SOD: superoxide dismutase; $^{**}p < 0.01$ vs. control; $^{****}p < 0.0001$ vs. control; ns: no significance.

levels have high diagnostic value in monitoring IR progression.

IR is a major pathogenetic factor in DM2 preceding the onset of overt hyperglycemia by up to several years [3]. Although decreased tissue sensitivity to insulin is compensated by hyperinsulinemia, IR leads to obesity, hypertension, dyslipidemia, and finally the metabolic syndrome [4]. Therefore, understanding the causes of IR and its treatment is one of the most significant challenges in modern diabetology. The gold standard for IR assessment is the hyperinsulinemic-euglycemic clamp technique [31]. However, this method is not used in routine medical practice due to its high invasiveness and the need to perform the test while the patient is hospitalized. For this reason, insulin resistance is mainly diagnosed using indirect methods. The simplest of them is a measurement of fasting glucose and insulin and calculation of HOMA-IR index (homeostatic model assessment of IR), which is a mathematical model describing interdependence of insulin secretion in response to current basal glycemia [3, 31]. Nevertheless, new biomarkers are still being sought that could noninvasively inform about the progression of IR and its metabolic complications [1]. Of particular diagnostic interest is saliva, which is easy to collect and does not require the specialized equipment or assistance of the medical staff. Saliva can be collected multiple times per day and can replace blood draws in people with clotting disorders, children, or patients with disabilities [24, 25]. Noninvasive saliva collection reduces patient anxiety, promotes more frequent self-monitoring, and enables the disease diagnosis at an early stage [32]. Unfortunately, the assessment of glucose and insulin in the saliva is not diagnostically relevant because it does not reflect their levels in the blood [33–35]. Nonetheless, there has been considerable recent interest in saliva-based diagnostics focusing on redox biomarkers. Salivary redox biomarkers are commonly used to diagnose hypertension [36, 37], obesity [38–40], chronic kidney disease [41, 42], heart failure [43, 44], Hashimoto's disease [45, 46], dementia [47, 48], or cancer [49, 50]. Considering the critical contribution of oxidative stress in the progression of IR [6, 9, 10, 15, 16], we evaluated the salivary antioxidants and products of protein and lipid oxidation/nitration in the saliva of IR rats. We compared the content of salivary redox biomarkers with their plasma levels and the classical indicators of metabolic disorders. We also assessed the diagnostic utility of salivary redox biomarkers using ROC analysis and multivariate regression.

We have shown that IR results in impairment of the salivary/plasma antioxidant barrier, with both enzymatic (↓SOD, ↓SPx/GPx) and nonenzymatic (↓GSH, ↓AA, ↑UA) deficiency. Although we did not assess the rate of ROS production, the weakening of antioxidant systems is likely due to increased free radical generation under IR conditions [44]. It is well known that positive energy balance (↑fat supply) causes increased synthesis of acetyl-CoA and NADP in mitochondria, responsible for ROS overproduction [51, 52]. However, adipose tissue is also an essential source of free radicals in IR [53]. Excess visceral tissue stimulates adipocytes to synthesize chemotactic and adhesive molecules such as MCP1 (monocyte chemoattractant protein 1), VCAM1 (vascular cell adhesion molecule 1), and ICAM (intercellular adhesion molecule 1), which enhance the influx of lymphocytes and macrophages and stimulate the production of proinflammatory cytokines (IL-1, IL-2, TNF-α) [53–55]. Synthesis of adipokines (resistin and leptin), which mediate inflammation by promoting cytokine efflux, is also increased [53]. Thus, it is not surprising that the antioxidant barrier is diminished, resulting in higher oxidative damage to proteins (↑PC, ↑3-NT, ↑AGE) and lipids (↑4-HNE). Of particular note are disturbances in glutathione metabolism (↓GSH, ↓SPx/GPx). GSH participates in hydrogen peroxide degradation and maintains the sulfhydryl groups of proteins in a reduced state [56]. The accumulation of oxidized glutathione (GSSG) in the cell and the formation of protein disulfides with GSH inhibits many enzymes' activity, thereby impairs cell metabolism and energy production [56, 57]. The increased oxidation (↑PC, ↑4-HNE), glycation (↑AGE), and nitration (↑3-NT) of proteins/lipids observed in our study are also very important. The products of oxidative/nitrosative modifications damage cellular structures and show



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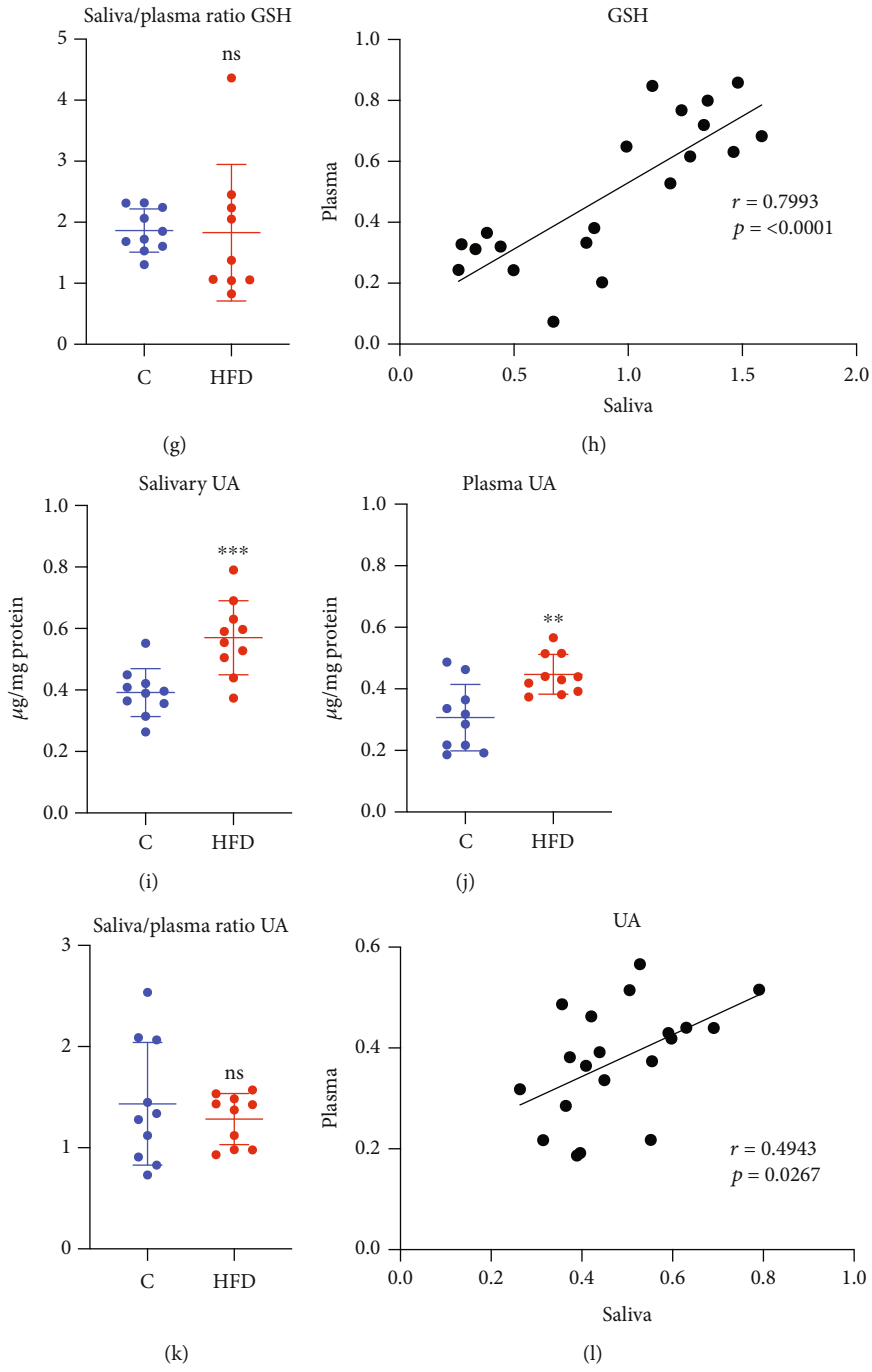
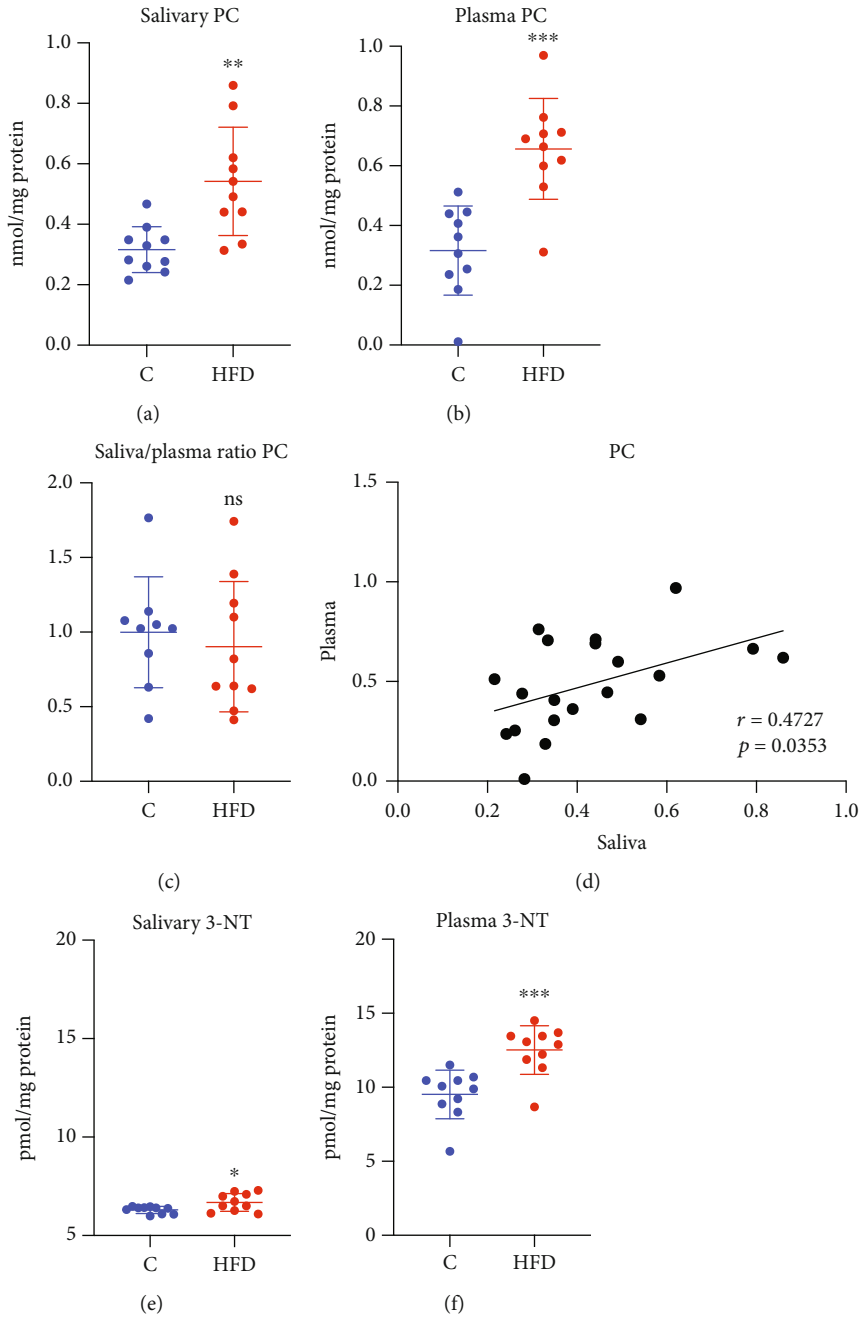


Figure 2: The effect of a high-fat diet (HFD) on salivary and plasma nonenzymatic antioxidants in rats. Abbreviations: C: control group; HFD: high-fat-diet group; AA: ascorbic acid; GSH: reduced glutathione; UA: uric acid; $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{***}p < 0.001$ vs. control; $^{****}p < 0.0001$ vs. control; ns: no significance.

mutagenic and carcinogenic properties. Indeed, compounds such as 4-HNE can form adducts with DNA, promoting instability of the genetic material and several replication errors [58, 59].

A crucial part of our study was to evaluate the diagnostic utility of salivary redox biomarkers in IR conditions. Biomarkers are biological indicators whose assessment allows qualitative or quantitative evaluation of pathological states and diseases. The biomarkers should differentiate patients from healthy controls (with high accuracy and specificity) and correlate with disease severity [23]. Of all the biomarkers we evaluated, salivary GSH and 4-HNE deserve special attention. Salivary GSH correlates negatively not only with body weight ($r = -0.667$, $p = 0.035$) and BMI ($r = -0.889$, $p = 0.001$), but also with plasma glucose ($r = -0.79$, $p = 0.006$), insulin ($r = -0.783$, $p = 0.007$), HOMA-IR ($r = -0.936$, $p = 0.00007$), and proinflammatory adipokines (leptin: $r = -0.757$, $p = 0.011$; resistin: $r = 0.685$,



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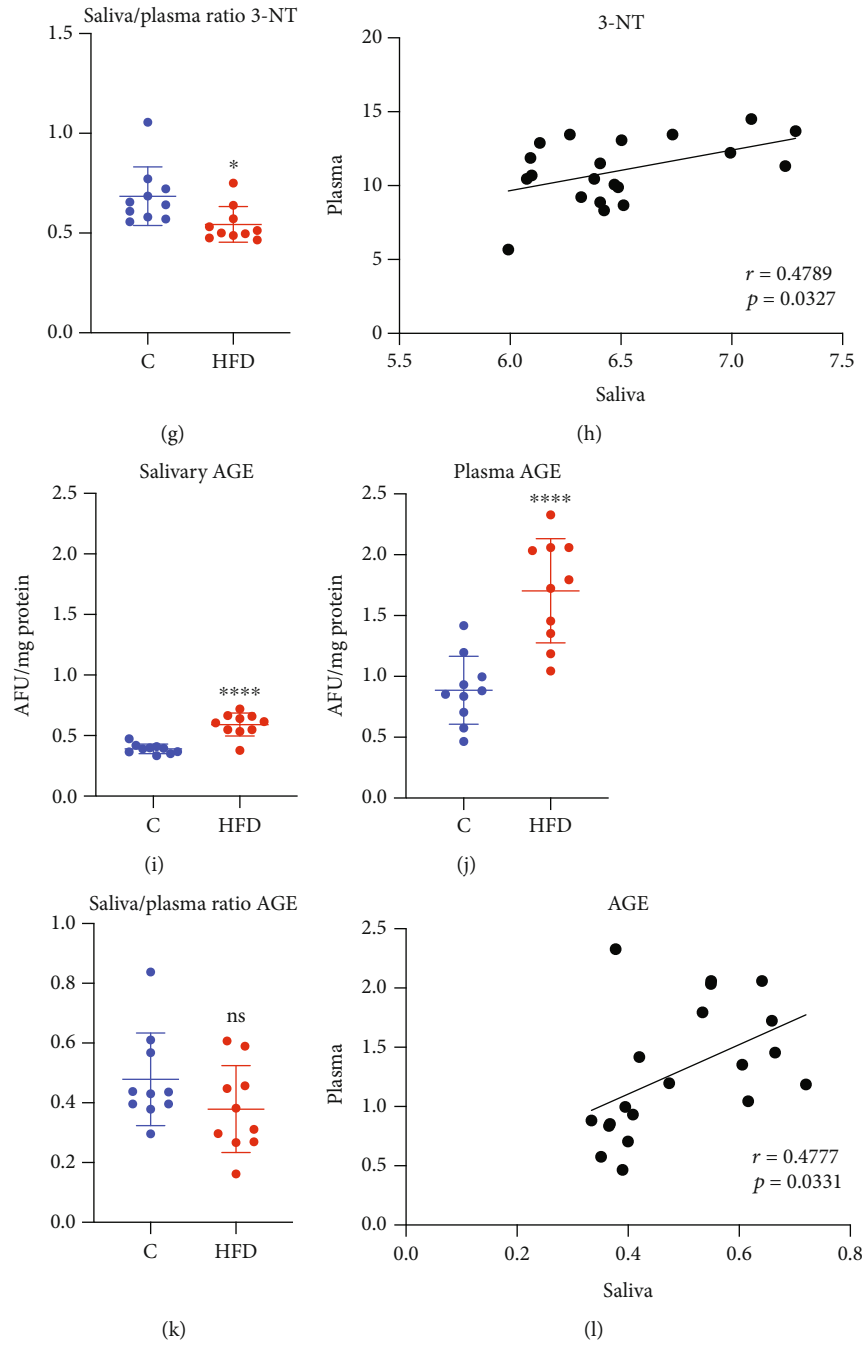

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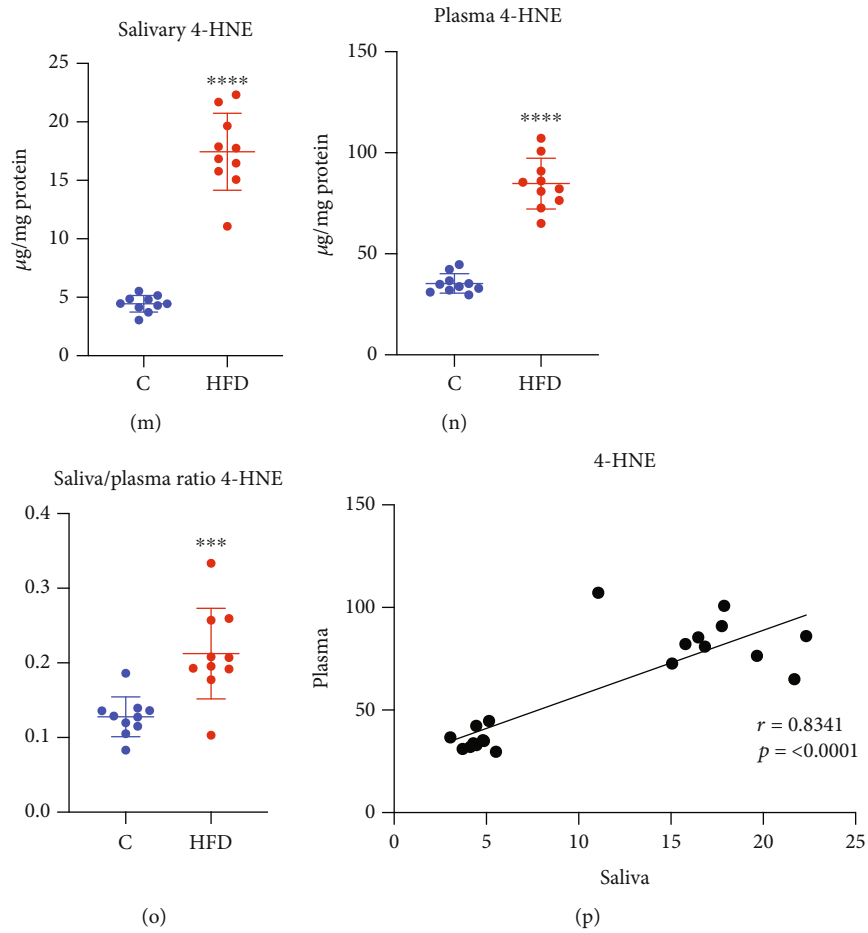

Figure 3: The effect of a high-fat diet (HFD) on salivary and plasma oxidative/nitrosative damage in rats. Abbreviations: C: control group; HFD: high-fat-diet group; 3-NT: 3-nitrotyrosine; 4-HNE: 4-hydroxynonenal; AGE: advanced glycation end products; PC: protein carbonyls; $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{***}p < 0.001$ vs. control; $^{****}p < 0.0001$ vs. control; ns: no significance.

$p = 0.029$; and TNF-α: $r = -0.639$, $p = 0.047$). Multivariate regression analysis also showed that salivary GSH depends on the severity of obesity measured by BMI and reduced insulin sensitivity expressed as HOMA-IR. Therefore, GSH depletion may be associated with the progression of metabolic disturbances accompanying insulin resistance. Indeed, glutathione is one of the most critical intracellular antioxidants [22, 60]. In addition to ROS scavenging and regenerating other antioxidants (e.g., vitamin E and GPx), GSH participates in restoring oxidatively modified proteins, lipids, and nucleic acids. It also acts as a major thiol buffer of the cell by regulating growth, differentiation, and apoptosis [56, 57]. Reduced GSH level is a critical factor in increasing the intensity of membrane lipid peroxidation and ceramide accumulation in patients with obesity and IR [22, 61, 62]. In our study, this may be supported by the negative correlation between salivary GSH and 4-HNE ($r = 0.663$, $p = 0.037$). Indeed, lipids are particularly susceptible to oxidation. Lipid peroxidation products such as 4-HNE cause further cellular damage, including disruption of gene expression/protein synthesis and uncoupling of oxidative phosphorylation [63]. 4-HNE can also increase inflammation by stimulating NADPH oxidase activity or activating macrophages [58, 64]. Therefore, the positive correlations of salivary 4-HNE and adipokines are not surprising (leptin: $r = 0.626$, $p = 0.053$; and resistin: $r = 0.776$, $p = 0.008$). However, salivary 4-HNE correlates also with body weight ($r = 0.605$, $p = 0.044$) and BMI ($r = 0.854$, $p = 0.002$), as well as indicators of carbohydrate metabolism (glucose ($r = 0.716$, $p = 0.02$); and HOMA-IR ($r = 0.592$, $p = 0.05$)). It is well known that IR is the most important cause of carbohydrate disorders. Hyperinsulinemia and insulin resistance are also independent factors in diabetes and cardiovascular disease [4, 5]. Therefore, we used multiple regression to determine the diagnostic utility of salivary 4-HNE in the HFD-induced IR model. Regression analysis showed that this parameter highly depends on the HOMA-IR index and BMI. Its positive correlation with proinflammatory adipokines also demonstrates the diagnostic utility of salivary 4-HNE.

The oral cavity is a unique site in the body since it is exposed to many prooxidant factors such as air pollutants, diet, medications, dental materials, and other xenobiotics [65, 66]. Although many antioxidants/oxidative stress products pass into saliva from the blood, their salivary content can not necessarily reflect the intensity of systemic oxidative

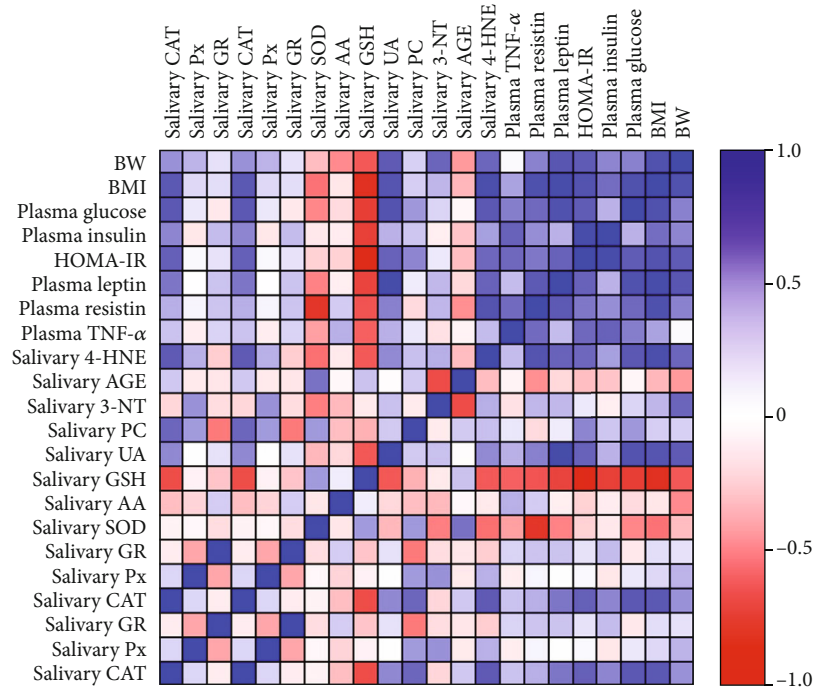

Figure 4: Correlations between salivary and plasma redox and metabolic parameters in rats. Abbreviations: 3-NT: 3-nitrotyrosine; 4-HNE: 4-hydroxynonenal; AA: ascorbic acid; AGE: advanced glycation end products; BMI: body mass index; BW: body weight; CAT: catalase; GSH: reduced glutathione; GR: glutathione reductase; HOMA-IR: homeostatic model assessment for insulin resistance; PC: protein carbonyls; Px: peroxidase; SOD: superoxide dismutase; TNF-α: tumor necrosis factor α; UA: uric acid.

Table 2: Multiple regression analysis of the analyzed redox biomarkers.

Parameter	β1: HOMA-IR			β2: BMI		
	Estimate	95% CI	p value	Estimate	95% CI	p value
Salivary CAT	0.03866	-0.1186 to 0.1959	0.5793	0.7877	-0.6970 to 2.272	0.2499
Salivary Px	-4.2	-23.23 to 14.83	0.6178	57.27	-122.3 to 236.9	0.4754
Salivary GR	0.07613	-1.587 to 1.740	0.9169	1.356	-14.35 to 17.06	0.844
Salivary SOD	0.02902	-0.02624 to 0.08427	0.2544	-0.4925	-1.014 to 0.02913	0.0607
Salivary AA	-1.039	-5.541 to 3.462	0.602	3.56	-38.93 to 46.05	0.8486
Salivary GSH	-0.2338	-0.3665 to -0.1011	0.0042	-1.44	-2.693 to -0.1877	0.0298
Salivary UA	0.01866	-0.1543 to 0.1916	0.806	1.175	-0.4573 to 2.808	0.1325
Salivary PC	0.2107	-0.1228 to 0.5443	0.1788	-0.9228	-4.071 to 2.226	0.5106
Salivary 3-NT	-0.2589	-1.162 to 0.6447	0.5198	4.142	-4.387 to 12.67	0.2885
Salivary AGE	-0.01719	-0.2152 to 0.1808	0.8432	-0.3393	-2.209 to 1.530	0.6806
Salivary 4-HNE	-0.9857	-4.660 to 2.688	0.546	47.96	13.29 to 82.64	0.0137

Abbreviations: 3-NT: 3-nitrotyrosine; 4-HNE: 4-hydroxynonenal; AA: ascorbic acid; AGE: advanced glycation end products; BMI: body mass index; CAT: catalase; CI: confidence interval; GSH: reduced glutathione; GR: glutathione reductase; HOMA-IR: homeostatic model assessment for insulin resistance; PC: protein carbonyls; Px: peroxidase; SOD: superoxide dismutase; UA: uric acid.

stress. Indeed, in our study, salivary antioxidant enzymes did not correlate with serum activity (exception: SOD). Nevertheless, salivary nonenzymatic antioxidants reflect very well their plasma levels. We also observed positive correlations between the concentrations of protein and lipid oxidation products in plasma and saliva of IR rats. However, the highest correlation coefficients are found for GSH ($r = 0.7993$, $p < 0.0001$) and 4-HNE ($r = 0.8341$, $p < 0.0001$). Therefore, salivary GSH and 4-HNE can be used to assess systemic redox homeostasis. The results of the ROC analysis also demonstrated the high diagnostic utility of these biomarkers. ROC analysis evaluates the diagnostic power of the test and assesses the ability of the biomarker to discriminate between normal and abnormal values. Salivary GSH and 4-HNE differentiate with very high sensitivity (100%) and specificity (100%) between healthy animals and those with IR (AUC = 1.0). Thus, salivary GSH and 4-HNE meet all the criteria for a good laboratory biomarker [23]. Further

Table 3: ROC analysis of the analyzed redox biomarkers.

Biomarker	Saliva								Serum/plasma							
	AUC	95% CI	p value	Cut-off	Sensitivity %	95% CI	Specificity %	95% CI	AUC	95% CI	p value	Cut-off	Sensitivity %	95% CI	Specificity %	95% CI
CAT	0.76	0.5360 to 0.9840	0.0494	>0.5592	60	31.27% to 83.18%	60	31.27% to 83.18%	1	1.000 to 1.000	0.0002	<1.244	100	72.25% to 100.0%	100	72.25% to 100.0%
Px/GPx	0.84	0.6581 to 1.000	0.0102	<29.80	70	39.68% to 89.22%	70	39.68% to 89.22%	0.63	0.3657 to 0.8943	0.3258	<35.10	50	23.66% to 76.34%	50	23.66% to 76.34%
GR	0.57	0.3009 to 0.8391	0.5967	<1.719	50	23.66% to 76.34%	50	23.66% to 76.34%	0.6	0.3419 to 0.8581	0.4497	<10.62	60	31.27% to 83.18%	60	31.27% to 83.18%
SOD	0.97	0.9071 to 1.000	0.0004	<0.2066	90	59.58% to 99.49%	90	59.58% to 99.49%	1	1.000 to 1.000	0.0002	<0.5534	100	72.25% to 100.0%	100	72.25% to 100.0%
AA	0.99	0.9583 to 1.000	0.0002	<9.841	90	59.58% to 99.49%	90	59.58% to 99.49%	0.89	0.7500 to 1.000	0.0032	<8.703	80	49.02% to 96.45%	80	49.02% to 96.45%
GSH	1	1.000 to 1.000	0.0002	<0.9395	100	72.25% to 100.0%	100	72.25% to 100.0%	1	1.000 to 1.000	0.0002	<0.4547	100	72.25% to 100.0%	100	72.25% to 100.0%
UA	0.9	0.7609 to 1.000	0.0025	>0.4447	80	49.02% to 96.45%	80	49.02% to 96.45%	0.86	0.6739 to 1.000	0.0065	>0.3867	80	49.02% to 96.45%	80	49.02% to 96.45%
PC	0.89	0.7476 to 1.000	0.0032	>0.3696	80	49.02% to 96.45%	80	49.02% to 96.45%	0.95	0.8477 to 1.000	0.0007	>0.4784	90	59.58% to 99.49%	90	59.58% to 99.49%
3-NT	0.78	0.5536 to 1.000	0.0343	>6.415	70	39.68% to 89.22%	70	39.68% to 89.22%	0.91	0.7557 to 1.000	0.0019	>11.01	90	59.58% to 99.49%	90	59.58% to 99.49%
AGE	0.94	0.8208 to 1.000	0.0009	>0.4471	90	59.58% to 99.49%	90	59.58% to 99.49%	0.95	0.8622 to 1.000	0.0007	>1.192	80	49.02% to 96.45%	80	49.02% to 96.45%
4-HNE	1	1.000 to 1.000	0.0002	>8.293	100	72.25% to 100.0%	100	72.25% to 100.0%	1	1.000 to 1.000	0.0002	>54.87	100	72.25% to 100.0%	100	72.25% to 100.0%

Abbreviations: 3-NT: 3-nitrotyrosine; 4-HNE: 4-hydroxynonenal; AA: ascorbic acid; AGE: advanced glycation end products; AUC: area under the curve; CAT: catalase; CI: confidence interval; GPx: glutathione peroxidase; GSH: reduced glutathione; GR: glutathione reductase; HOMA-IR: homeostatic model assessment for insulin resistance; PC: protein carbonyls; Px: peroxidase; ROC: receiver operating characteristic; SOD: superoxide dismutase; UA: uric acid.

clinical studies are needed to evaluate their diagnostic potential. However, it should not be forgotten that in IR there is salivary hypofunction caused by disturbances in redox homeostasis [12, 67, 68], which may affect the oxidative stress parameters in saliva. It is also necessary to compare salivary redox biomarkers in patients with IR and those with other metabolic, cardiovascular, and inflammatory diseases [44].

It is essential to note the limitations of our work. Because of the lack of Ethics Committee approval, we could not perform a hyperinsulinemic-euglycemic clamp. Additionally, we evaluated only the most commonly assessed redox biomarkers due to the low volume of saliva. Although the redox biomarkers described here distinguish IR rats and controls with high specificity and sensitivity, they may be nonspecific only for insulin resistance. Therefore, it is essential to evaluate salivary redox indicators also in other diseases with oxidative stress etiology. Nevertheless, this is the first study to show the potential use of saliva and oxidative stress biomarkers in monitoring IR progression.

4. Conclusions

(1) Saliva can be used to assess the systemic antioxidant status and the intensity of systemic oxidative stress

(2) Salivary GSH and 4-HNE may be potential biomarkers of IR progression

(3) There is a need for human clinical trials to evaluate the diagnostic utility of salivary redox biomarkers in IR conditions

5. Materials and Methods

5.1. Animals. The experiment was performed on male Wistar rats (*R. norvegicus*; Wistar: cmd, outbred Cmdb:Wi) with an initial body weight of 50–60 g. The animals came from the Center for Experimental Medicine of the Medical University of Bialystok. The rats have been housed in individually ventilated laboratory cages at controlled temperatures (20–22°C), under a standard condition of light from 6.00 a.m. to 6.00 p.m., and with free access to tap water and food.

The experimental procedures were approved by the institutional Committee for Ethics use of Animals in the University of Warmia and Mazury in Olsztyn, Poland (No. 21/2017).

After seven days of adaptation, the rats were divided into two groups of 10 individuals each.

(i) Group I—(C) control; rats receiving standard rodent diet (Research Diets, New Brunswick, NJ, USA, catalog number D12450J) containing 10% fat, 20% proteins, and 70% carbohydrates

(ii) Group II—(HFD) rats fed a high-fat diet (Research Diets, New Brunswick, NJ, USA catalog number D12492) containing 60% fat, 20% proteins, and 20% carbohydrates

Animals from control and HFD groups were fed the appropriate diet for eight weeks, while the body weight and food intake were monitored weekly. The body mass index (BMI) was calculated using the formula BMI = body weight (g)/length^2 (cm^2), and rat length was measured from the tip of the nose to the anus. BMI between 0.45 and 0.68 g/cm^2 was considered normal values, whereas BMI greater than 0.68 g/cm^2 indicated obesity [69, 70].

After eight weeks of the experiment, rats were fasted for 12 h, anesthetized with sodium phenobarbital (80 mg/kg body weight, intraperitoneally), and then the whole saliva was collected. The animals were peritoneally injected with 5 mg/kg BW pilocarpine nitrate (Sigma Chemical Co; St. Louis, MO, USA) in physiological saline. Five minutes later, a preweighted cotton ball was inserted into the oral cavity, and saliva was collected for five minutes [67, 68]. The volume of saliva was evaluated by subtracting the initial weight of cotton balls from their final weight. One mg of the collected saliva was considered to be one μL [67, 68]. Saliva was then centrifuged in Salivette tubes (3000 × g, 4°C, 10 min) to collect supernatant [71]. Next, whole blood was collected from the abdominal aorta into glass tubes (to obtain serum) and EDTA tubes (to obtain plasma) and centrifuged (3000 × g, 4°C, 10 min). To protect against sample oxidation and proteolysis, the antioxidant butylated hydroxytoluene (BHT, ten μL of 0.5 M BHT in acetonitrile per 1 mL sample; Sigma-Aldrich, Steinheim, Germany) and a protease inhibitor (Complete Mini Roche, France) were added to the collected saliva and plasma samples [72, 73]. All samples were stored at -80°C but for no longer than six months.

Fasting blood glucose level was determined using the glucometer (Accu-Chek; Bayer, Germany). Fasting plasma insulin level was determined using a commercial ELISA kit according to the manufacturer's instructions (EIAab Science Inc. Wuhan; Wuhan, China). To confirm IR, the insulin sensitivity was determined using the homeostasis model assessment (HOMA − IR) = fasting insulin (U/mL) × fasting glucose (mM)/22.5 [74]. According to the manufacturer's instructions, plasma adipocytokines (leptin, resistin, and TNF-α) were determined using a commercial ELISA kit (EIAab Science Inc. Wuhan; Wuhan, China).

5.2. Redox Assays

5.2.1. Enzymatic Antioxidants. The activity of catalase (CAT) was analyzed spectrophotometrically by measuring the decomposition rate of hydrogen peroxide (H_2O_2) in the sample at 240 nm wavelength [75]. One CAT unit was expressed as the amount of enzyme that decomposes 1 mmol H_2O_2 within 1 minute. Salivary peroxidase (Px) activity was determined spectrophotometrically according to Mansson-Rahemtulla et al. method [76]. The absorbance changes in the reaction mixture containing 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), potassium iodide (KI), and H_2O_2 were measured at 412 nm wavelength. The activity of glutathione peroxidase (GPx) was assayed spectrophotometrically by the Paglia and Valentine method [77]. The absorbance was analyzed at 340 nm, based on the conversion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to reduced nicotinamide adenine dinucleotide phosphate (NADP+). One unit of GPx activity was assumed to catalyze

the oxidation of 1 mmol of NADPH for 1 minute. The method of Mize and Langdon [78] was used to assess glutathione reductase (GR) activity. The absorbance of the samples was measured at 340 nm wavelength. One unit of GR activity was expressed as the amount of enzyme needed for the oxidation reaction of 1 μmol of NADPH within 1 minute. The activity of superoxide dismutase (SOD) was determined spectrophotometrically by measuring the absorbance changes accompanying adrenaline oxidation at 480 nm wavelength [79]. One unit of SOD activity was assumed to inhibit the oxidation of adrenaline by 50%.

5.2.2. Nonenzymatic Antioxidants. The concentration of ascorbic acid (AA) was analyzed colorimetrically according to Jagota and Dani [80]. This method involves the reduction of the Folin phenol reagent under the influence of AA. The absorbance of the samples was measured at 760 nm wavelength. The content of reduced glutathione (GSH) was measured spectrophotometrically based on the reduction of DTNB to 2-nitro-5-mercaptobenzoic acid under the influence of GSH. The absorbance was measured at 412 nm wavelength [81]. The concentration of uric acid (UA) was analyzed colorimetrically by measuring the absorbance of 2,4,6-tripyridyl-s-triazine complex with iron ions and UA, using the commercial kit QuantiChromTM Uric Acid DIUA-250 (BioAssay Systems, Harward, CA, USA). The intensity of the examined sample was measured at 490 nm wavelength.

5.2.3. Oxidative/Nitrosative Damage. The concentration of protein carbonyl (PC) was determined colorimetrically based on the 2,4-dinitrophenylhydrazine (2,4-DNPH)'s reaction with carbonyl groups in the oxidatively damaged proteins. The intensity of the resultant hydrazone was measured at 355 nm. PC content was calculated using an absorption coefficient for $2,4-\mathrm{DNPH} = 22{,}000\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$. According to the manufacturer's instructions, the concentration of 3-nitrotyrosine (3-NT) was measured by the ELISA method, using a commercial diagnostic kit (Immundiagnostik AG; Bensheim, Germany). Advanced glycation end product (AGE) level was analyzed spectrofluorimetrically by measuring the specific AGE fluorescence at 350/440 nm [82]. The samples were diluted in 0.02 M PBS buffer for the AGE determination in plasma [44]. The concentration of 4-hydroxynonenal protein adduct (4-HNE) was determined by the ELISA method (OxiSelect™HNE Adduct Competitive ELISA Kit, Cell Biolabs Inc. San Diego, CA, USA), following the manufacturer's instructions provided in the package.

5.3. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 8.4.3 for MacOS (GraphPad Software, La Jolla, USA). The Shapiro–Wilk test was used to determine the normality of distribution, while the Student's *t*-test was used to compare the IR group with the controls. The results were presented as mean ± standard deviation (SD), and the value of $p < 0.05$ was considered statistically significant. Pearson correlation coefficient was used to evaluate the relationships between redox biomarkers and metabolic parameters. To identify factors that determine the levels of redox biomarkers, we performed multiple regression analyses. HOMA-IR and BMI were included as independent variables; 95% confidence intervals (CI) were reported along with regression parameters. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic utility of the redox biomarkers. AUC (area under the curve) and optimal cut-off values were determined for each parameter that ensured high sensitivity with high specificity.

The number of animals was calculated a priori based on our previous preliminary study. Type I error $\alpha = 0.05$ and statistical power (type II error) of 0.9 were considered. Statistical test assumptions were validated for all the analyses performed. The minimum number of rats in one group was seven, and therefore, the analysis was performed on ten individuals.

Data Availability

The datasets generated for this study are available on request to the corresponding author.

Ethical Approval

The study was approved by the institutional Committee for Ethics use of Animals in the University of Warmia and Mazury in Olsztyn, Poland (No. 21/2017).

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization was done by M.M. and A.Z.; data curation was done by M.M. and A.Z.; formal analysis was done by M.M.; funding acquisition was done by M.M. and A.Z.; investigation was done by M.M.; methodology was done by M.M. and C.P.; material collection was done by M.M. and M.Z.P.; supervision was done by J.R.L. and A.Z.; validation was done by M.M.; visualization was done by M.M.; writing—original draft was done by M.M.; writing—review and editing was done by M.M. and A.Z. All authors have read and agreed to the published version of the manuscript.

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Review Article

Redox-Dependent Effects in the Physiopathological Role of Bile Acids

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Bile acids (BA) are recognized by their role in nutrient absorption. However, there is growing evidence that BA also have endocrine and metabolic functions. Besides, the steroidal-derived structure gives BA a toxic potential over the biological membrane. Thus, cholestatic disorders, characterized by elevated BA on the liver and serum, are a significant cause of liver transplant and extrahepatic complications, such as skeletal muscle, central nervous system (CNS), heart, and placenta. Further, the BA have an essential role in cellular damage, mediating processes such as membrane disruption, mitochondrial dysfunction, and the generation of reactive oxygen species (ROS) and oxidative stress. The purpose of this review is to describe the BA and their role on hepatic and extrahepatic complications in cholestatic diseases, focusing on the association between BA and the generation of oxidative stress that mediates tissue damage.

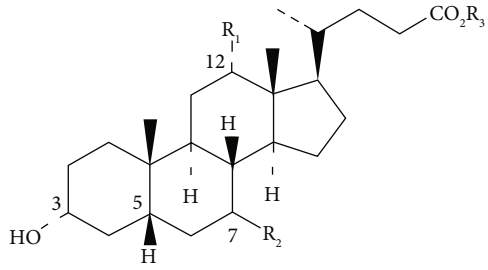
1. Introduction

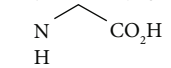
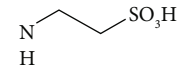
Bile acids (BA) are a group of steroidal molecules derived from cholesterol. These molecules have been historically described as solubilizing agents for lipids and activators for pancreatic enzymes, supporting their role in intestinal absorption [1, 2]. While the BA are intrinsically toxic in elevated concentrations due to the amphipathic structure, several antecedents indicate that BA also have endocrine and metabolic functions. Furthermore, despite their steroidal nature, the BA stereochemistry differs from other steroids, such as steroidal hormones. Therefore the receptor and signaling vary [3, 4].

Cholestatic liver diseases and the complications derived from the gradual destruction of bile ducts produce BA accumulation in the liver. This increment of BA induces a proinflammatory response and an increased production of reactive oxygen species (ROS), leading to cellular damage. Cholestatic pathologies do not have effective treatments, making them one of the leading causes of liver transplants [5–7].

Several pathological conditions, endogenous or xenobiotic-induced, might generate the obstruction of bile flow, elevating the BA concentrations within hepatocytes and serum and damaging the neighboring tissues [6, 8, 9]. In this line, the BA-dependent cytotoxicity and cellular alterations are associated with oxidative stress, mainly affecting the liver and extrahepatic tissues such as the heart, skeletal muscle, and placenta. In the central nervous system (CNS), contradictory effects of BA and their receptors reportedly show generation or prevention of oxidative stress [10–12].

Table 1: The general structure of more abundant bile acids in humans.



Bile acid	R1	R2	R3
Cholic acid	OH	α-OH	Acid form
Chenodeoxycholic acid	H	α-OH	H
Deoxycholic acid	OH	H	Glycine-conjugate
Lithocholic acid	H	H	
Ursodeoxycholic acid	H	β-OH	Taurine-conjugate 

This review presents a description of BA and their primary receptors, their clinical association with cholestatic diseases, and the impact of BA-induced oxidative stress observed in critical tissues.

2. Bile Acids

BA are amphiphilic molecules that belong to the acidic sterol family. They have a unique stereochemistry, hydroxyl groups, and an aliphatic side chain with a terminal carboxyl residue. All hydroxyl groups and the side carboxyl group are faced in the same plane, except in ursodeoxycholic acid (UDCA) (Table 1), forming a structure with opposing lipophilic properties [13]. BA correspond to the bile's significant lipidic component and are synthesized from cholesterol in the liver and secreted to store in the gallbladder [1, 13]. *De novo* synthesized BA, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are categorized as primary and are the most abundant species in humans. The primary BA can be conjugated with glycine or taurine at the side chain, increasing the water solubility before secretion into the canalicular duct. After the release into the small intestine, primary BA can be dehydroxylated by the intestinal microbiota, converting CA and CDCA into the secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Also, the 7-hydroxyl group in CDCA can be epimerized to form the UDCA [4, 13–15].

The BA form micelles (in concentrations between 1 and 20 μM) with hydrophobic compounds, facilitating absorption processes at the intestine. Besides, lipid absorption is favored by BA-dependent pancreatic lipase activation [14, 16]. Then, unconjugated and conjugated BA are reabsorbed in the small intestine and colon via passive and active transport back to the liver, completing the enterohepatic circulation [13, 16].

Further, BA have endocrine/metabolic functions, regulating their synthesis, transport, and detoxification; mediating the cellular energetics and lipid and glucose homeostasis; and modulating the intestinal microbiota [7, 13, 14, 16, 17].

Alterations in BA metabolism and transport lead to pathological conditions. For example, high levels of BA in enterohepatic circulation can damage the liver and intestine, generating jaundice, cholesterol gallstones, and cholestatic liver diseases. Conversely, BA deficiency leads to nutrient malabsorption and fat-soluble vitamin deficiency [1, 7, 13, 18]. Both extreme situations highlight the importance of a balanced BA metabolism due to their significant role in corporal homeostasis.

3. Bile Acid Receptors

The amphipathic nature of BA has been used to describe their significant physiological properties. However, the metabolic role of bile acids has been described mainly by discovering diverse receptors [1].

The BA receptors can be classified into two major groups: the nuclear and G-protein-coupled receptors. Below, we focused on the most widely described receptors, the farnesoid X receptor (FXR) and Takeda-G-protein-receptor-5 (TGR5) receptor, mentioning other receptors with a lesser expression and minor characterization in the literature (Table 2).

3.1. Farnesoid X Receptor. Initially, the FXR was recognized as a receptor for farnesol and some related metabolites. It forms a heterodimeric complex with the retinoid X receptor. In 1999, it was reported that BA are the physiologic ligands of FXR by three independent groups. The ligand-receptor interaction is independent of the conjugation status of BA, whereas the affinity of this interaction is determined by the substitutions in carbon 7 of BA [19–21]. FXR is encoded

Table 2: Bile acid receptor distribution and ligands.

Receptor	Classification	Distribution	Main agonist	References
FXR	Nuclear receptor	Liver and intestine Minor expression in the heart, kidneys, CNS, white adipose tissue, adrenal gland, pancreas, and placenta	CDCA > LCA = DCA > A 7α-OH ≫ 7-keto ≫ 7β-OH	[4, 20, 69]
PXR	Nuclear receptor	Liver and intestine Kidney, stomach, and CNS	LCA ≈ CDCA ≈ CDA	[61, 64]
CAR	Nuclear receptor	Liver, kidney, CNS, and adrenal gland	LCA	[65]
VDR	Nuclear receptor	Small intestine, colon, skin, heart, and kidney	LCA and metabolite	[67, 70]
TGR5	G-protein-coupled receptor	Heart, skeletal muscle, lung, spleen, kidney, liver, CNS, enteric nervous system, gastrointestinal tract, placenta, and adipocytes	LCA > DCA > UDCA > CDCA > CA	[10, 40, 46, 47, 71]
S1PR2	G-protein-coupled receptor	Liver, small intestine, CNS, and enteric nervous system	Conjugated DCA ≈ conjugated CA	[72, 73]

by the *fxr* gene that generates four transcripts' variants, all responsive to BA [22]. Another gene in mammals, *fxr*β (pseudogene in humans and primates), expresses the FXRβ receptor that senses mainly lanosterol, and to a minor extent, BA [23].

FXR is involved in the metabolism and regulation of BA levels. Thus, FRX diminishes BA synthesis by repressing the critical enzyme expression associated with this process, such as cytochrome (CYP) 7a1 and 12-α-hydroxylase [3, 24–26]. Also, FXR decreased intracellular levels of BA in hepatocytes by two mechanisms: downregulating the uptake transporters (SLCA1 and SLCO1A2) and upregulating the levels of efflux transporters (BSEP, MRP2, and OSTα) [27–30]. Furthermore, FXR diminishes the intestinal absorption of BA by inhibiting the expression of apical sodium-dependent BA transporter, an uptake transporter from enterocytes in the ileum, colon, and jejunum [31].

FXR is also related to a protective effect in several tissues. For example, the absence of FXR expression has been associated with vacuolization and hepatocyte hypertrophy, and also with increased serum triglyceride, cholesterol, glucose, and BA (resulting in mild cholestasis) [3, 32–34]. Also, the absence of FXR expression affected cardiac function and elevated the levels of myocardial injury markers associated with a BA overload [35]. Similarly, in a diabetes mice model, the FXR knock-out aggravates cardiac fibrosis and lipid accumulation [36]. Furthermore, FXR agonists diminish cardiac fibrosis, kidney damage, and pancreatic hypertrophy and reduce lipid serum levels in obese/diabetic mice models, decreasing hepatic fibrosis and portal pressure in a nonalcoholic steatohepatitis rat model [37–39]. These antecedents demonstrate the importance of FXR on corporal function via homeostasis of BA, carbohydrates, and lipids.

3.2. TGR5. The primary membrane receptor for BA is the G-protein-coupled TGR5, also called BG37, GPBAR1, or M-BAR. There is a correlation between BA's hydrophobicity and affinity for TGR5. Besides, the TGR5 activity associates with elevated intracellular calcium levels and cytosolic cyclic adenosine monophosphate (cAMP), independently of FXR activation [40, 41].

TGR5 couples mainly with G(s) protein in several tissues [42–46]. However, paradoxical effects were observed in subtypes of cholangiocytes. In ciliary cholangiocytes, TGR5 agonists diminish cAMP levels and induce the extracellular signal-regulated kinase (ERK) signaling pathway. Still, in nonciliary cholangiocytes, TGR5 activation increased the cAMP levels and inhibited the ERK pathway, subsequently activating proliferation [42, 47]. Also, TGR5 activation has been associated with the induction of other signaling pathways, such as AKT/mTOR and NF-κB [48–50].

The metabolic effects are associated with TGR5 activation. In the gastrointestinal tract, TGR5 activation induces the expression of glucagon-like peptide-1, mediating glucose homeostasis and the BA prokinetic effect [51, 52]. Besides, TGR5 activation increases the energy expenditure in brown adipose tissue by a mechanism dependent on type 2 iodothyronine deiodinase [43, 53].

Recently, our group demonstrated that DCA and CA, in a TGR5-dependent manner, induced sarcopenia and atrophy in skeletal muscle by incrementing the ubiquitin-proteasome system (UPS) and oxidative stress [44]. Also, the absence of the TGR5 receptor prevents the sarcopenia induced by cholestatic chronic liver disease, protecting the muscle from loss of mass and strength [54]. These results contradict a report indicating that TGR5 enhances muscle differentiation in the C2C12 myoblast and induces hypertrophy in mice [45]. These studies differ in the knock-out mice model and the used BA, suggesting that more analyses are necessary to understand the effect of BA in skeletal muscle and the importance of conjugation- and hydrophobicity-specific effect.

3.3. Other Bile Acid Receptors. The sphingosine-1-phosphate receptor 2 (S1PR2) senses the phosphorylated sphingosine and mediates mainly cell proliferation and differentiation. This membrane receptor has a high affinity to conjugated BA [55, 56]. S1PR2 activation induces the phosphorylation of ERK1/2 and AKT and reduces the BA-induced apoptosis in hepatocytes by preventing intracellular calcium oscillations [56, 57]. S1PR2 also activates the NF-κB pathway through EGFR/ERK1/2/AKT, inducing a proinflammatory

response [58, 59]. Besides, the absence of S1PR2 favors the development of fatty liver during a high-fat diet in mice through the sphingosine kinase 2 [60]. These antecedents suggest that BA may modulate lipid metabolism in the liver through S1PR2.

The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are intracellular sensors that mediate the detoxification process of xenobiotics [61, 62]. These receptors can bind BA and modulate the expression of genes involved in BA metabolism [63]. In this way, BA activates PXR and CAR, increasing the expression of enzymes (e.g., CYP3A, CYP2B, and sulfotransferases) that modify BA, reducing their hydrophobicity to decrease their toxicity. Besides, PXR and CAR generate diverse isoforms of BA's efflux transporters (MRP and OATP), increasing the clearance of hydrophobic BA [61, 64–66]. Therefore, both receptors complement the function of FXR by decreasing the toxicity and increasing the excretion of BA to protect the tissues from citotoxicity.

Also, the vitamin D receptor (VDR) can sense the LCA and its metabolites, but not other BA. Furthermore, VDR induces the expression of CYP3A on the small intestine and MRP3 in the colon [67, 68]. These reports suggested that VDR is a sensor that mediates the protection of the intestinal tract from toxic LCA levels.

To summarize, membrane receptors, such as TGR5 and S1PR2, are mainly associated with BA-dependent endocrine/metabolic functions in diverse tissues, unlike nuclear receptors directly related to BA homeostasis.

4. Bile Acid Cytotoxicity

The lipophilicity of BA is directly proportional to their cytotoxic effect due to their potential to solubilize and disrupt cell membranes. Cellular swelling, apoptosis, alterations in membrane integrity, and release of several cellular components are characteristic of BA-induced toxicity [9, 74–76]. In addition, due to the general structure, BA could induce lipid peroxidation and alterations in the lipid composition of membranes [77, 78].

In addition to membrane alterations, hydrophobic BA induce a proinflammatory response in hepatocytes by increasing membrane adhesion molecules and chemokines [79, 80]. Also, CDA and its conjugated derivatives can activate the caspase pathway in a Fas receptor-dependent mechanism [81, 82]. These antecedents indicate that BA can induce a proinflammatory response and facilitate cell death (Figure 1).

The mitochondrial function is severely affected by elevated BA levels [9, 83]. Lipophilic BA decrease the state 3 respiration and the membrane potential in mitochondria from the liver and the heart [74, 77, 84]. BA also induce the permeability transition pore and favor the release of cytochrome C into the cytosol, associated with the enhanced expression and translocation of Bax to mitochondria together with the decreased Bcl-2 expression [83, 85–88]. Furthermore, most hydrophobic BA increase mitochondrial hydroperoxide and the accumulation of compounds derived from lipid peroxidation [89]. Nevertheless, a recent report suggests that mitochondrial toxicity does not precede cytotoxicity. Other mechanisms such as lipid membrane disruption or ROS generation explain BA-dependent cytotoxicity [9]. These antecedents suggest that mitochondria are a primary target affected by BA and can be a source of oxidative stress through alterations in the electron-transport chain, favoring the cytotoxic effect (Figure 1).

It has been widely described that increased levels of lipophilic BA can induce apoptosis in diverse cell lines and tissues. However, not all BA are associated with cell damage [69, 74, 76, 90]. Particularly UDCA, the more hydrophilic BA, prevents hepatic damage by inhibiting the JNK signaling pathway and controlling the location of proapoptotic protein Bax at the mitochondrial membrane [75, 91]. Moreover, UDCA prevents the apoptosis induced by other molecules such as ethanol, TGF-β1, or Fas ligand, avoiding mitochondrial dysfunction and releasing cytochrome C [74, 92]. Nevertheless, coincubation of UDCA and CDCA shifts apoptosis to necrosis as the predominant cell death route in cultured human hepatocytes [83]. Similarly, the taurine-conjugated UDCA reduces the DNA fragmentation and mitochondrial dysfunction induced by ischemia in rat brains and inhibits mitochondrial efflux of cytochrome C through PI3K signaling pathway activation in rat cortical neurons [93, 94]. Also, tauro-UDCA reduces apoptosis by preventing the increase of caspase-12/Bax and the endoplasmic reticulum stress via AKT activation in mice with brain injury [95, 96].

In summary, BA can alter membranes, affecting cell structures, such as membrane and mitochondria. Besides, BA induce oxidative stress and proinflammatory response and also activate cell death pathways (Figure 1). All these mechanisms are closely associated with the structural properties of BA and have been used to explain their cytotoxicity.

5. Redox-Dependent Mechanisms Participate in Damage Induced by Bile Acids

The intracellular milieu is in a constant equilibrium between production and degradation of reactive oxygen, nitrogen, iron, copper, and sulfur species, generally named ROS [97]. A balanced ROS production is fundamental to normal cell function [98, 99]. ROS can be divided into radical (superoxide anion or hydroxyl radical) and nonradical species (hydrogen peroxide or hypochlorous acid, among others). ROS can be generated through enzymatic or nonenzymatic reactions [100, 101]. The intracellular oxidant species can be counterbalanced by systems that neutralize the electrophilic properties of ROS. These systems include catalase, glutathione-S-transferase, superoxide dismutase (SOD), and nonenzymatic molecules such as glutathione, thioredoxin, or vitamin E [102, 103].

Oxidative stress is established by a disturbance between ROS and antioxidants that results in excessive oxidant milieu, leading to cellular injury [97]. Oxidative stress damages cell structures by modifying proteins, lipids, nucleotides, and membranes, affecting their functions and limiting cell viability [102, 104]. To characterize and quantify oxidative stress, the ROS levels and antioxidant activity are

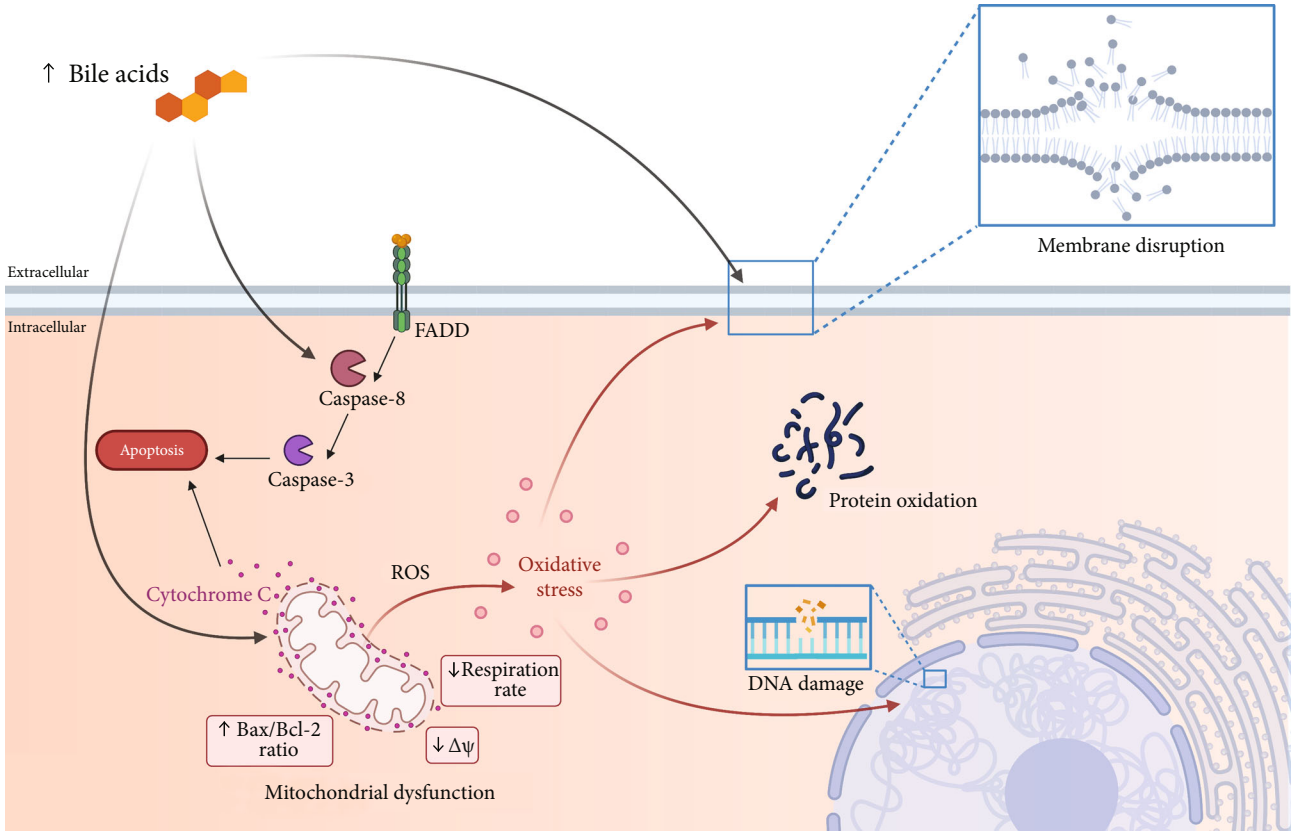


Figure 1: Cytotoxicity mechanisms induced by bile acids (BA). BA can induce membrane disruption by an alteration of stability and composition due to their steroid structure. Moreover, BA activate the caspase pathway in a Fas receptor-dependent mechanism (FADD), triggering cellular apoptosis. In addition, BA affect the mitochondrial function by (1) decreasing the rate of respiration, (2) diminishing the membrane potential, (3) increasing the permeability transition pore facilitating the translocation of cytochrome C and contributing to apoptosis, and (4) inducing reactive oxygen species (ROS) generation. The increased ROS levels lead to cellular oxidative stress capable of inducing DNA damage, protein oxidation, and lipid peroxidation, contributing to cellular membrane damage. All these mechanisms impair cellular viability.

typically determined. In addition, other parameters are end products of the oxidative modification such as lipid peroxidation (malondialdehyde (MDA), thiobarbituric acid-reactive substances (TBARS), 4-hydroxy-2-nonenal (4-HNE) or F2-isoprostanes), protein oxidation (carbonylated proteins), or even DNA oxidation (8-hydroxy-2′-deoxyguanosine (8-OHdG)) [103].

Below, we will detail the main effects of oxidative stress in the tissues most affected by cholestatic disorders (Figure 2).

5.1. Liver. Hepatocytes are highly affected by elevated BA levels [80]. Experiments in hepatocytes showed that lipophilic BA (CDCA, DCA, and CA) increase cellular hydroperoxide, superoxide anion, and TBARS production [74, 75, 89]. Also, the taurine conjugates of CDCA and CA increase the MDA levels and correlate with a decline in hepatocyte viability. This cellular toxicity was prevented by different antioxidant mechanisms [105]. These antecedents suggest that BA-induced oxidative stress affects hepatocyte viability.

FXR regulates BA homeostasis through diverse mechanisms, explaining the predominant role on cholestasis etiology [8, 26, 106]. In this line, the absence or inhibition of FXR results in a high BA concentration in serum and promotes hepatic injury [3, 69]. The *Fxr*-null mice showed an increased hepatic BA concentration causing an elevation of oxidative markers such as 8-OHdG, hydroperoxide, and TBARS. Besides, these mice also increased protective Nrf2 signaling in hepatic tissue, probably to counterbalance the cellular damage [33].

Other reports using a rat model fed with a BA-supplemented diet or a bile duct ligation model showed swollen mitochondrial and impaired cellular respiration, both associated with elevated ROS production [74, 75]. Together, these results suggest that high serum concentrations of BA induce hepatic oxidative stress.

5.2. Skeletal Muscle. Extrahepatic dysfunctions characterize cholestatic hepatic diseases. Among them are weakness and skeletal muscle wasting. This complex syndrome is named sarcopenia. Among the features of sarcopenia is the decreased cross-sectional area of muscle fibers due to several molecular mechanisms such as diminished protein synthesis, high protein degradation, mitochondrial dysfunction, dysregulated autophagy, and oxidative stress [104, 107, 108].

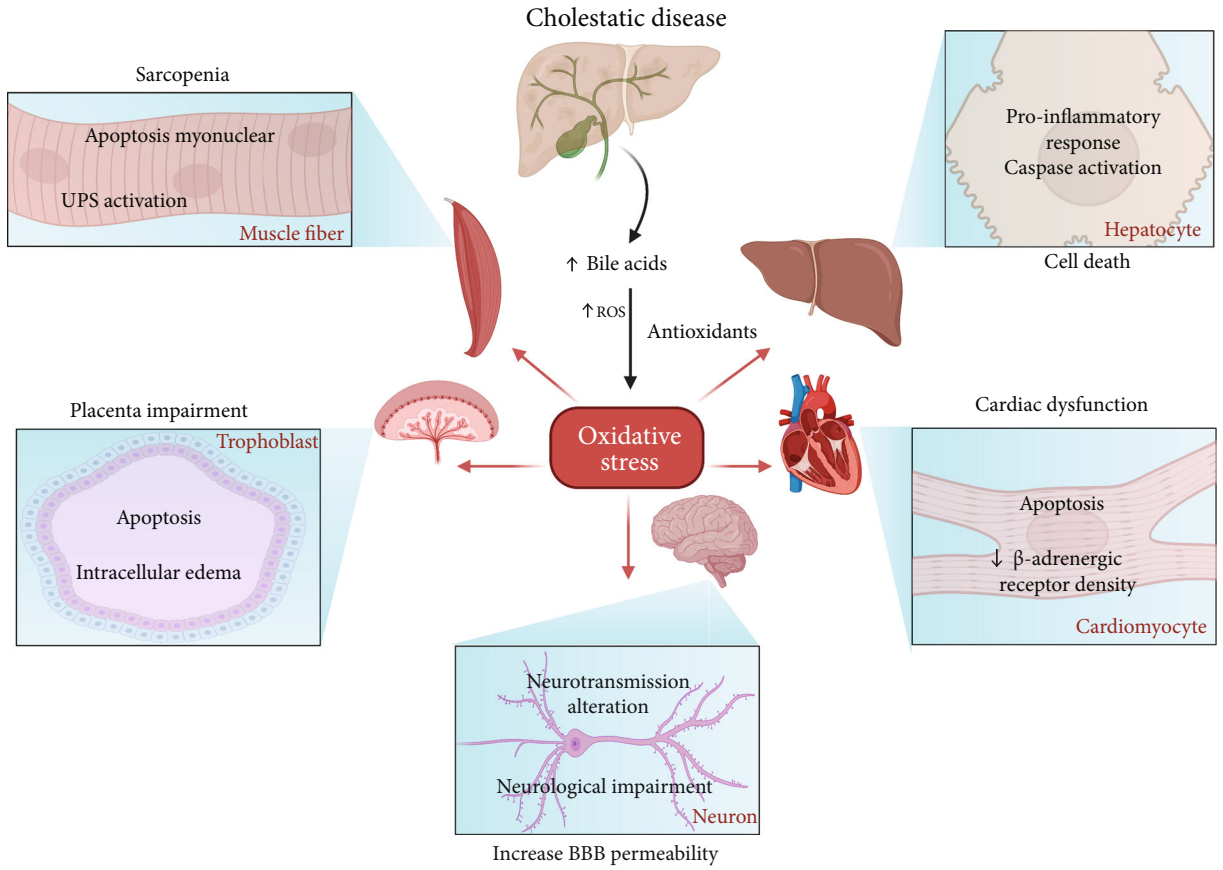


Figure 2: The effect of bile acid-induced oxidative stress in different tissues during a cholestatic disease. Cholestatic conditions provoke elevated serum levels of BA. Consequently, there is an imbalance between reactive oxygen species (ROS) and antioxidant systems, leading to oxidative stress. In skeletal muscle, sarcopenia is caused due to ubiquitin-proteasome system (UPS) activation and myonuclear apoptosis in fibers. In hepatic tissue, oxidative stress mediated a proinflammatory induction and caspase activation in hepatocytes. Besides, oxidative stress induces apoptosis and intracellular edema in the trophoblast during pregnancy, causing impairment in the placenta. Elevated BA levels increase the blood-brain barrier (BBB) permeability and correlated with neurological impairment and altered neurotransmission. Finally, oxidative stress induces cardiac dysfunction through apoptosis and a reduction in β-adrenergic receptor density in cardiomyocytes.

Our laboratory described the induction of sarcopenia in a mice model of cholestatic liver disease characterized by TGR5-dependent mechanisms: (1) oxidative stress, presenting elevated ROS, carbonylated proteins, and 4-HNE in skeletal muscles; (2) increased myonuclear apoptosis, with induction of the caspase pathway and increased Bax/Bcl-2 ratio; and (3) induction of protein catabolism through UPS [54, 107, 109]. Interestingly, ROS is directly associated with the UPS induction and mitochondrial alterations that might induce apoptosis [108, 110–112]. In addition, the use of an antioxidant treatment (N-acetyl cysteine) prevents muscle damage and diminishes the apoptotic effect [109].

Moreover, recently it has been described that CA and DCA resemble the skeletal muscle atrophy induced by cholestatic liver disease, UPS induction, and oxidative stress. Also, the absence of TGR5 in muscle fibers abolished all harmful effects caused by these BA [44]. Thus, all these antecedents firmly suggest that elevated BA in cholestatic disorders induce oxidative stress through the TGR5 receptor, activating several intracellular events that cause sarcopenia.

A recent study has shown a relationship between muscle-BA-gut microbiota. Results indicate that the alteration of gut microbiota induced sarcopenia. This muscle dysfunction was associated with an altered profile of BA that reaches the portal blood circulation. This change induces the inhibition of ileal FXR signaling with the consequent decrease in serum levels of FGF15, an enterokine related to muscle wasting [113]. Considering the antecedents related to muscular TGR5, BA, and sarcopenia [44, 54], it is impossible to discard this receptor's participation in the muscle dysfunction associated with alteration in the microbiota-BA axis.

5.3. Central Nervous System. Oxidative stress is crucial in hepatic encephalopathy, and altered BA levels (elevated, changes on conjugated/unconjugated and primary/secondary ratio) could be associated with neurological decline [104, 114–116]. It was described that BA, via Rac1 activity, increase the blood-brain barrier permeability, facilitating the neurological changes associated with cholestatic diseases

[116]. Also, patients with Alzheimer's disease present with increased secondary and conjugated BA levels that correlate with the disease's advanced stages [117]. Furthermore, CA, DCA, and CDCA modulate the respiratory-related rhythmic discharge activity in an FXR-dependent manner, which interferes with NMDA or GABA neurotransmission, suggesting that BA affects the brain's normal function [118, 119].

The FXR and TGR5 receptors have been associated with neurological damage. Downregulation of FXR in the frontal cortex replicated the neuroprotective effect of reducing BA levels in mice with acute liver failure, suggesting that FXR signaling mediates the neurological decline in this model [114]. Additionally, the absence of FXR correlates with reduced brain infarct volume, prevents neuronal apoptosis by an anti-inflammatory response, and reduces calcium influx after oxygen-glucose deprivation in a cerebral ischemia mice model [120]. Further, the TGR5 receptor in astrocytes responds to neurosteroids, molecules with structural similarities with BA, elevating the intracellular calcium and ROS [10]. Those results confirm that the BA receptors could be relevant in generating oxidative stress and neurological impairment.

Nevertheless, tauro-UDCA prevents lipopolysaccharide depressive-like mice model, an effect that correlated with neuroinflammatory protection and decreased MDA/nitrite levels in the hippocampus and prefrontal cortex [121]. Similarly, the hydrophobic CA induced anti-inflammatory properties and reduced oxidative stress (decreasing MDA, NO, Il-1β, and TNF-α) in an integrative functional unit composed of neurons and neural supporting cells known as the neurovascular unit [12]. The prevention of oxidative stress and neuroprotective effect might be related to the TGR5 receptor. Its activation with a semisynthetic agonist decreased oxidative stress and neuronal apoptosis and downregulated the NF-κB pathway in mice with brain injury [37, 71]. Those results suggest that BA might have different roles in the oxidative stress induction in CNS in noncholestatic conditions.

Considering oxidative stress with neurological pathologies and the conflicting description of BA on the oxidative stress in CNS, it is crucial to perform more mechanistic analysis to understand BA's role. Also, understanding the BA-CNS relation raises the possibility of proposing novel pharmacological strategies, including BA receptor modulation, for neurological disorders and neurodegenerative pathologies.

5.4. Heart. During cholestatic diseases, serum BA elevation is associated with direct toxic effects on the heart and the impairment of myocardial function [35, 90, 122]. In addition, CDCA induces apoptosis in neonatal rat ventricular myocytes due to the loss of mitochondrial membrane potential and cytochrome C release, as well as consequent caspase-pathway activation. The bile duct ligation model resembles the cardiac proapoptotic response and shows an impairment contractibility [82, 87].

Also, CA decreases the heart rate and myocardial contraction and increases the markers of cardiac injury concomitantly with decreased β-adrenergic receptor density. These characteristics resemble the cardiac alteration in the FXR knock-out model and cholestatic liver disease [35, 123]. Interestingly, FXR inhibition suppresses cardiac apoptosis. Additionally, FXR inhibition in an ischemia-reperfusion model reduces cardiotoxicity and decreases myocardial infarct size improving cardiac function [87]. However, a contradictory report showed that FXR agonists activate the Nrf2 signaling (decreasing ROS, MDA, and 8-OHdG through elevated catalase, glutathione-S-transferase, and SOD), preventing cardiomyopathy in a diabetic mice model [124]. Those results suggest that BA directly and via oxidative stress could mediate the cardiotoxicity. However, the protective effect of FXR must be analyzed deeply.

Furthermore, BA also activate TGR5 in ventricular myocyte cell culture [46]. Selective TGR5 agonist (INT-777) prevents NF-κB activation and decreases the ROS level induced by high glucose treatment in primary cardiomyocytes [125]. Moreover, LCA prevents high glucose-induced hypertrophy in the cardiac myoblast cell line, and TGR5 upregulation alleviates the oxidative stress and inflammatory process through activating the AKT pathway in the cardiac myoblast cell line [126–128]. Also, the TGR5-dependent protective effect induced by BA *in vivo* was described with the administration of DCA in a cardiac injury mice model, improving cardiac remodeling and inhibiting the proinflammatory response [128]. These results suggest that TGR5 has a protective role in myocardial tissue associated with diminishing oxidative stress.

In general, BA can exert opposing effects on the myocardial tissue depending on the mediated receptor involved. All those results indicate that BA can be one of the responsible causes of cardiac impairment by several mechanisms in cholestasis. However, TGR5 showed a promissory pharmacological target.

5.5. Placenta. Intrahepatic cholestasis in pregnancy increases the risk of adverse outcomes, even causing intrauterine death [69]. The placenta has a protective role to the fetus from molecules of different structural nature. During pregnancy, the increased serum BA impaired the protective function of the placenta and enhanced the toxicity to the fetus [86, 129]. Studies using trophoblast cell lines and diverse gestational cholestatic animal models showed edema and apoptosis in the placenta, attenuating with FXR agonist or UDCA treatments [69, 86, 130, 131]. Also, UDCA has been successfully proved in intrahepatic cholestasis in pregnancy patients without interfering with the placental hormone production and with no-fetal side effects. However, it does not enhance the perinatal death ratio, BA concentration, and itch score [132–134].

The elevated BA levels are associated with the increase of oxidative stress markers (MDA and carbonylation proteins) in the placenta, as well as the decrease of antioxidant gene expression and activity of catalase, glutathione-S-transferase, SOD, peroxiredoxin (PRDX), among others [69, 86]. Also, increased MDA levels and diminished expression of PRDX1 and PRDX3 were reported in the placenta from intrahepatic cholestasis of pregnant human patients [69]. These results suggest that oxidative stress induced by BA mediates the

placenta's impairment and contributes to the affectation in the mother and fetus.

6. Clinical Perspective and Conclusions

BA are amphiphilic molecules mainly characterized by their ability to form micelles, and they are associated with nutrient absorption at the intestinal level. However, BA have endocrine functions that regulate metabolic activity and cellular energy through facilitating lipid- and carbohydrate metabolism. Several receptors are associated with BA-dependent actions, such as FXR, TGR5, S1PR2, PXR, CAR, and VDR. Indeed, FXR and TGR5 have been widely studied to understand BA effects and propose novel therapeutics for cholestatic disorders.

The FXR receptor has a central role in BA physiology and carbohydrate and lipid homeostasis. Most cholestatic disorders are characterized by BA transport impairments associated with FXR malfunction, making this receptor an attractive molecular target to treat cholestasis [1, 32, 135]. Also, it has been reported that UDCA decreased FXR activation and increased triglycerides in obese patients [136]. This evidence enhances the interest in developing FXR activators. Interestingly, some non-BA molecules that can activate FXR have been tested in preclinical studies [137–140].

Conversely, the TGR5 receptor has a pivotal role in cell differentiation in some cell lines, and its activation is also associated with diverse signaling pathways [42, 48]. Also, TGR5 activation was associated with upregulation of type 2 iodothyronine deiodinase, increased production of glucagon-like peptide-1, and even intestinal motility [43, 52, 141, 142]. Since the TGR5 functions are related to metabolism and there exists the need for treating metabolic diseases such as diabetes or obesity, there is an increased interest in finding novel TGR5 agonists [5, 143–145].

Due to the relevance on metabolism and gastrointestinal physiology, BA receptors have been studied as a pharmacological target to treat some diseases. Indeed, some BA such as UDCA, CA, DCA, and CDCA, have been clinically approved by the U.S. Food and Drug Administration (FDA) to treat some pathological conditions. For example, they can dissolve and prevent gallstone (UDCA), primary biliary cirrhosis (UDCA, CDCA, and obeticholic acid), BA synthesis disorders (CA), and more recently, they have been used to improve the appearance of submental fat (DCA) [146, 147].

Despite these antecedents, BA or modified BA are still under clinical research to approve their therapeutic indication. The potential use of BA as a treatment for pathologies has been established in phase I clinical trials. Thus, TUDCA and CDCA administration improves insulin sensitivity through increased glucagon-like peptide-1 secretion in patients with obesity and diabetes. Besides, UDCA administration induces hepatic-protective properties after radiation [148–151]. A combination of taurine-UDCA and phenylbutyrate demonstrated prevention of functional decline and prolonged survival in patients with amyotrophic lateral sclerosis [152, 153]. Together, these clinical studies suggest that BA have promising effects on nongallbladder pathologies. Nevertheless, more advanced clinical trials are needed to demonstrate that BA can be used in these conditions and the eventual relationship with oxidative stress.

Treatment with BA generates unwanted side effects such as diarrhea/excessive flatus and pruritus. Interestingly, modified BA and non-BA FXR agonists are helpful to prevent those adverse effects. The diarrhea is associated with alteration in secretion and motility in the colon, and activation of FXR by obeticholic acid or tropifexor (non-BA FXR agonist) increases the feedback inhibition via fibroblast growth factor 19, improving diarrhea scores [154, 155].

Although several clinical trials with BA failed to improve nonalcoholic steatohepatitis, the obeticholic acid improved hepatic histology, decreased fibrosis, and increased insulin sensitivity [156–158]. Also, obeticholic acid reduced serum alkaline phosphatase level in patients with primary biliary cholangitis [159]. However, similar to other BA, obeticholic acid developed pruritus in patients in different clinical trials [156, 159, 160]. The beneficial effect without this secondary effect was obtained by using 24-*nor*-UDCA in patients with primary sclerosing cholangitis or nonalcoholic fatty liver disease [157, 161]. These reports suggest that modified BA could be the better option for future treatments. However, long-term studies with modified BA are needed to analyze the relevance of side-effect prevention, as well as the relation with oxidative stress.

Furthermore, the agonism of TGR5 or FXR could be inappropriate in other tissues, mainly in the skeletal muscle, heart, and gallbladder, presenting some adverse effects [162–164]. Recently, it has been reported that obeticholic acid may increase the gallstone risk by a mechanism dependent on FXR activation and FGF19 participation [165]. However, it is essential considering the severe side effects, mainly with long-term and high-dose BA treatments, as UDCA was associated with increased risks of developing colorectal neoplasia in primary sclerosing cholangitis, and its withdrawal deteriorates liver serum markers and increases pruritus [166–168].

More recently, there is an interest in developing FXR/TGR5 dual agonists due to an eventual synergistic effect [169]. Some beneficial effects have been reported in preclinical studies of kidney disease and liver steatosis through anti-inflammatory mechanisms [170–173]. However, there are only initial reports, and additional research is necessary to establish the relevance of this dual strategy.

The cellular alterations induced by elevated BA levels include membrane damage, proinflammatory response, mitochondrial dysfunction, and cell death by apoptosis or necrosis. All of these effects are directly or indirectly related to redox-dependent mechanisms. Interestingly, in hepatic tissues, oxidative stress and cellular damage are closely associated with FXR signaling. Meanwhile, in skeletal muscle, BA-induced injury is a TGR5-dependent process. In all mentioned tissues in this review (hepatic, skeletal muscle, CNS, heart, and placenta), oxidative stress has a significant role in apoptosis. However, an evidence gap indicates that additional research must be performed to understand and establish the complex signaling involved in the potential harm of the BA-oxidative stress axis and the long-term effect of BA as a therapeutical

option. In the same direction, BA-induced redox signaling is a central hallmark that could be considered a target for developing innovative therapeutic options to treat cholestatic diseases.

Abbreviations

4-HNE: 4-Hydroxy-2-nonenal
8-OHdG: 8-Hydroxy-2′-deoxyguanosine
AKT: Protein kinase B
BA: Bile acids
Bax: Bcl-2-associated X
Bcl-2: B-cell lymphoma 2
BSEP: Bile salt export pump
CA: Cholic acid
cAMP: Cyclic adenosine monophosphate
CAR: Constitutive androstane receptor
CDCA: Chenodeoxycholic acid
CNS: Central nervous system
CYP: Cytochrome
DCA: Deoxycholic acid
DNA: Deoxyribonucleic acid
EGFR: Epidermal growth factor receptor
ERK: Extracellular signal-regulated kinases
FXR: Farnesoid X receptor
LCA: Lithocholic acid
LPS: Lipopolysaccharide
MDA: Malondialdehyde
MRP: Multidrug resistance proteins
mTOR: Mammalian target of rapamycin
NF-κB: Nuclear factor kappa beta
OATP: Organic anion transporting polypeptide
OST: Organic solute transporter
PRDX: Peroxiredoxin
PXR: Pregnane X receptor
ROS: Reactive oxygen species
S1PR2: Sphingosine-1-phosphate receptor2
SLC: Solute carrier
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid-reactive substances
TGF-β1: Transforming growth factor-beta type 1
TGR5: Takeda-G-protein-receptor-5
UDCA: Ursodeoxycholic acid
UPS: Ubiquitin-proteasome system
VDR: Vitamin D receptor.

Data Availability

Data is available on request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Research Article

Protective Activity of Aspirin Eugenol Ester on Paraquat-Induced Cell Damage in SH-SY5Y Cells

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Aspirin eugenol ester (AEE) is a new pharmaceutical compound esterified by aspirin and eugenol, which has anti-inflammatory, antioxidant, and other pharmacological activities. The aim of this study was to investigate the protective effect of AEE on paraquat- (PQ-) induced cell damage of SH-SY5Y human neuroblastoma cells and its potential molecular mechanism. There was no significant change in cell viability when AEE was used alone. PQ treatment reduced cell viability in a concentration-dependent manner. However, AEE reduced the PQ-induced loss of cell viability. Flow cytometry, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and 4′6-diamidino-2-phenylindole (DAPI) staining were used to evaluate cell apoptosis. Compared with the PQ group, AEE pretreatment could significantly inhibit PQ-induced cell damage. AEE pretreatment could reduce the cell damage of SH-SY5Y cells induced by PQ via reducing superoxide anion, intracellular reactive oxygen species (ROS), and mitochondrial ROS (mtROS) and increasing the levels of mitochondrial membrane potential ($\Delta\Psi m$). At the same time, AEE could increase the activity of glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) and decrease the activity of malondialdehyde (MDA). The results showed that compared with the control group, the expression of p-PI3K, p-Akt, and Bcl-2 was significantly decreased, while the expression of caspase-3 and Bax was significantly increased in the PQ group. In the AEE group, AEE pretreatment could upregulate the expression of p-PI3K, p-Akt, and Bcl-2 and downregulate the expression of caspase-3 and Bax in SH-SY5Y cells. PI3K inhibitor LY294002 and the silencing of PI3K by shRNA could weaken the protective effect of AEE on PQ-induced SH-SY5Y cells. Therefore, AEE has a protective effect on PQ-induced SH-SY5Y cells by regulating the PI3K/Akt signal pathway to inhibit oxidative stress.

1. Introduction

Parkinson's disease (PD) is a typical age-related chronic progressive disease, and it is also one of the most common neurodegenerative diseases [1]. PD primarily results from the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [2]. Clinically, PD is associated with motor impairments including bradykinesia, rigidity, resting tremor, and gait disturbance [3]. However, the mechanism remains elusive. Studies showed that oxidative stress, impairment of mitochondrial, and apoptotic cascade activation play important roles in the occurrence and development of PD [4, 5]. Similarly to other neurodegenerative diseases, PD patients display increased levels of oxidative stress and ROS, decreased mitochondrial membrane potential, and activated caspase cascade [6, 7].

Although the causes of PD are still unclear, the evidence strongly suggests that mitochondrial dysfunction and oxidative stress are involved in its pathogenesis [8]. Factors that increase the risk of PD have been identified, including increased age, exposure to environmental toxins, and genetic factors [9, 10]. These factors affect the function of the mitochondria through oxidative stress and then lead to neuronal apoptosis [11]. Among them, environmental factors are considered to play the key role in neurotoxicity, especially factors leading to oxidative stress, such as pesticides or heavy metals

[12]. PQ is a highly toxic nonselective herbicide that is widely used throughout the world [13–15]. Epidemiological studies showed that acute PQ poisoning can lead to severe brain damage and increase the incidence of PD [16]. Some studies showed that long-term low-dose exposure to PQ could induce the formation of α-synuclein aggregates, which could change the catabolism of dopamine and inactivate tyrosine hydroxylase [17, 18].

There is still lack of neuroprotective drug for treatment of neurodegenerative diseases; some drugs are used to attenuate symptoms [19–22]. Aspirin provided a clear neuroprotection against tetrahydropyridine toxicity on the striatal and nigral levels in murine Parkinson's model [19]. Aspirin had a protective effect on nerve injury induced by 1-methyl-4-phenylpyridiniumion and 6-hydroxydopamine in rats [22]. The combined use of docosahexaenoic acid and aspirin could significantly promote the expression of neurotrophic factors and promote the formation of PPARα and RXRα heterodimer, which provides a new method for the treatment of PD [21]. NOSH-aspirin, a novel nitric oxide and hydrogen sulfide-releasing hybrid, attenuates neuroinflammation induced by microglial and astrocytic activation [20]. Similarly, eugenol also has a neuroprotective effect [23, 24]. The main component of grassleaf sweetflag rhizome is eugenol, which could cross the blood-brain barrier and protect neurons. Studies have shown that eugenol has a neuroprotective effect on PC12 cells induced by amyloid-beta42 [24]. Behavioral and biochemical results showed the neuroprotective effects of eugenol and isoeugenol on acrylamide-induced neuropathy in rats [23].

As a new compound, AEE plays an active role in many aspects [25–34]. AEE has not only the effects of anti-inflammation, antithrombosis, and antiblood stasis but also the effect of antiatherosclerosis and other cardiovascular diseases. It is not clear whether AEE can play a neuroprotective role in neurodegenerative diseases. The purpose of this study was to explore whether AEE can attenuate PQ-induced oxidative damage in SH-SY5Y cells and its possible mechanism.

2. Materials and Methods

2.1. Chemicals. Aspirin eugenol ester (purity 99.5%) was prepared in Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS (Lanzhou, China). Dimethyl sulfoxide was supplied by Sigma (St. Louis, MO). Methyl viologen dichloride was purchased from Aladdin (Shanghai, China). Dulbecco's modified Eagle medium and fetal bovine serum were from Gibco (Grand Island, NY, USA). One Step TUNEL apoptosis assay kit, puromycin dihydrochloride, bicinchoninic acid assay kit, glutathione peroxidase kit, catalase assay kit, DAPI staining solution, and DAF-FM diacetate kit were obtained from Beyotime (Shanghai, China). Anti-PI3K, anti-Akt, anti-phosphorylation-PI3K (Tyr458), and anti-phosphorylation-Akt were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-Bax, anti-Bcl-2, and anti-caspase-3 were from Abcam (Cambridge, MA, USA). An Annexin V/FITC apoptosis detection kit was from BD Biosciences (San Diego, CA, USA). PI3-kinase inhibitor LY294002 was purchased from MedChemExpress LLC (New Jersey, USA). Lipofectamine™ 3000 transfection reagent was purchased from Thermo Fisher (Invitrogen, USA). Lentivirus control and PI3K shRNA (U6-MCS-Ubiquitin Cherry-IRES-puromycin) were purchased from GeneChem (Shanghai, China).

2.2. Cell Cultures and Cell Treatment. SH-SY5Y human neuroblastoma cells were routinely maintained in 10% fetal bovine serum (FBS), 1% GlutaMAX, 1% sodium pyruvate, 1% nonessential amino acids (NEAA), 87% 1 : 1 mix of F12, and modified Eagle medium (MEM) media supplemented with 2 mM L-glutamine at 37°C under humidified atmospheric conditions containing 5% CO_2. Media were replaced every two days. Subcultures were performed with the trypsin-EDTA method. Experiments were subsequently conducted on 6-7 passages of cells. SH-SY5Y human neuroblastoma cells were randomly divided into three groups ($n = 6$): control group, PQ group, and AEE pretreatment group. Cells in the control group were incubated with the normal growth conditions. Those in the PQ group were incubated with the medium containing 250 μM of PQ for 24 h. In the AEE pretreatment groups, the cells were cultured with the medium containing different concentrations of AEE (1, 2.0, and 4.0 μM) for 24 h before they were incubated with medium containing 250 μM PQ for 24 h.

2.3. Cell Viability. The viability of SH-SY5Y cells was detected by using a cell counting kit-8 (CCK-8) following the instructions of the manufacturer [28].

2.4. Flow Cytometric Analysis. SH-SY5Y cells (1×10^5/well) were seeded in 6-well plates. After treatments, cells were assessed using the Annexin V/FITC apoptosis detection kit according to the manufacturer's protocols [28]. The cells were sorted by a flow cytometer (BD FACSVerse, CA, USA), and the data were analyzed with FlowJo 7.6. For proper statistical analysis, more than 10000 cells per group were counted, and each assessment was repeated three times.

2.5. Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$). The $\Delta\Psi m$ was determined using MitoTracker® Red CMXROS (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, the cells (1×10^4/well) were seeded in 12-well plates. MitoTracker® Red probe was directly added into the culture media and incubated for 30 min at 37°C in the dark. Images were captured using a scanning laser confocal microscope (LSM800; Carl Zeiss, Germany).

2.6. Measurement of Intracellular and Mitochondrial Reactive Oxygen Species (ROS) and Superoxide Anion. Intracellular and mitochondrial ROS generation, and superoxide anion were measured using a DCFH-DA or MitoSOX™ red probe or dihydroethidium (DHE) as described previously method [35].

2.7. DAPI and TUNEL Staining. SH-SY5Y cells (1×10^4/well) were seeded into 12-well culture plates. After treatment, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS at 25°C for 30 min. After the cells were washed with PBS twice, 0.3% Triton X-100 PBS was added and incubated at 25°C for 5 min. TUNEL detection solution was added.

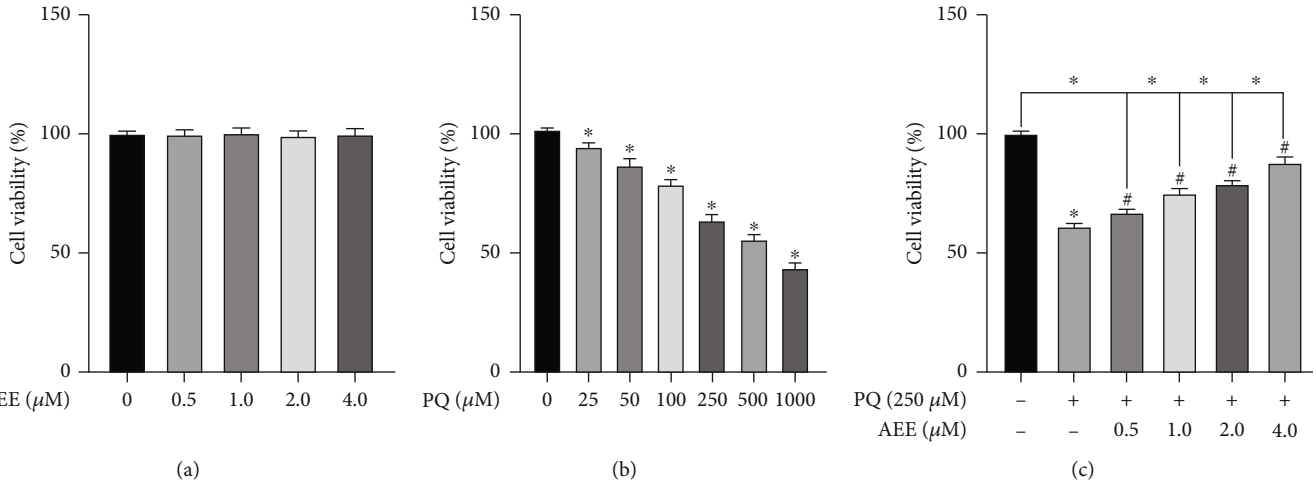


Figure 1: Protective effects of AEE on the cell viability of PQ-induced SH-SY5Y cells. (a) Different concentrations of AEE had no effect on the viability of SH-SY5Y cells. (b) PQ induced a concentration dependent decreased in SH-SY5Y cell viability. (c) AEE could significantly inhibit the decrease of SH-SY5Y cell viability induced by PQ. Values are presented as the means ± SD where applicable ($n = 6$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells.

After incubation of cells at 37°C for 1 h, DAPI staining solution was added and incubated at room temperature for 20 min. The cells were washed with PBS. Images were captured using a scanning laser confocal microscope (LSM800, Carl Zeiss, Germany). The TUNEL index (%) was based on the fluorescence microscopy detection of TUNEL-positive cells and DAPI-positive cells.

2.8. Determination of MDA, SOD, GSH-Px, and CAT in SH-SY5Y Cells. The activities of MDA, SOD, GSH-Px, and CAT in SH-SY5Y cells were assessed using the corresponding commercial kits according to the manufacturer's protocols [36, 37].

2.9. Protein Expression Analysis. The expressions of Bcl-2 (1 : 1000), Bax (1 : 5000), caspase-3 (1 : 500), PI3K (1 : 1000), Akt (1 : 1000), phospho-PI3K (1 : 1000), and phospho-Akt (1 : 1000) were assessed by western blot analysis. Protein was extracted from SH-SY5Y cells with RIPA lysis buffer containing 1 mM PMSF and a cocktail of protease and phosphatase inhibitors. The protein concentration was quantified using a bicinchoninic acid (BCA) assay kit. All measured proteins were normalized to β-actin level, and later, p-protein/total protein was calculated. Protein samples were separated by SDS-PAGE using 4-20% precast gradient polyacrylamide gels (Shanghai Suolaibao Bio-Technology Co., Ltd., Shanghai, China). After separation by SDS-PAGE, proteins were transferred to a PVDF membrane. The results were detected using G:BOX Chemi XRQ imaging system (Cambridge, Britain). ImageJ software (NIH) was used for quantification of band intensities, and intensities were normalized with total protein or the load control. Three duplicate wells were set in each group for the assay.

2.10. Cell Transfection. Lentiviral vectors expressing PI3K shRNA or control shRNA were obtained from GeneChem (Shanghai, China). Following the manufacturer's protocol, SH-SY5Y cells were cotransfected with lentivirus and packaging vectors using Lipofectamine 3000. Lentiviruses were harvested 48 h after transfection, centrifuged, and filtered through 0.45 μm membrane filters (Millipore). Lentiviruses were transduced in 50% confluent SH-SY5Y cells. Stable clones were selected for 1 week by using puromycin (1 μg/mL).

2.11. Determination of Apoptosis after Inhibition of Signal Pathway. To further examine the role of the PI3K/Akt signal pathway in AEE inhibiting PQ-induced apoptosis in SH-SY5Y cells, the PI3K signaling pathway in the SH-SY5Y cells was inhibited by shRNA and inhibitor LY 294002 against PI3K. In this part, it was divided into eleven groups. These SH-SY5Y cells were treated with 4.0 μM AEE and PQ according to the protocol described in Section 2.2.

2.12. Statistical Analysis. Statistical analysis was carried out using the SAS 9.2 (SAS Institute Inc., NC, USA). All data are presented as the means ± SD. The differences among different treatment groups were analyzed with one-way ANOVA followed with Duncan's multiple comparisons. Statistical significance was considered at $p < 0.05$. All experiments were performed as three independent replicates.

3. Results

3.1. AEE Protects the Cell Viability of PQ-Stimulated SH-SY5Y Cells. AEE (0.5, 1, 2, and 4 μM) alone had no significant effect on the viability of SH-SY5Y cells (Figure 1(a)). The SH-SY5Y cells were treated to various concentrations of PQ (25, 50, 100, 250, 500, and 1000 μM/mL) for 24 h. The results showed that treatment with PQ reduced cell viability in a concentration-dependent manner. When the cells were treated with 250 μM PQ for 24 h, the cell viability decreased significantly compared with the control group (Figure 1(b)). Therefore, the concentration of 250 μM PQ was used in

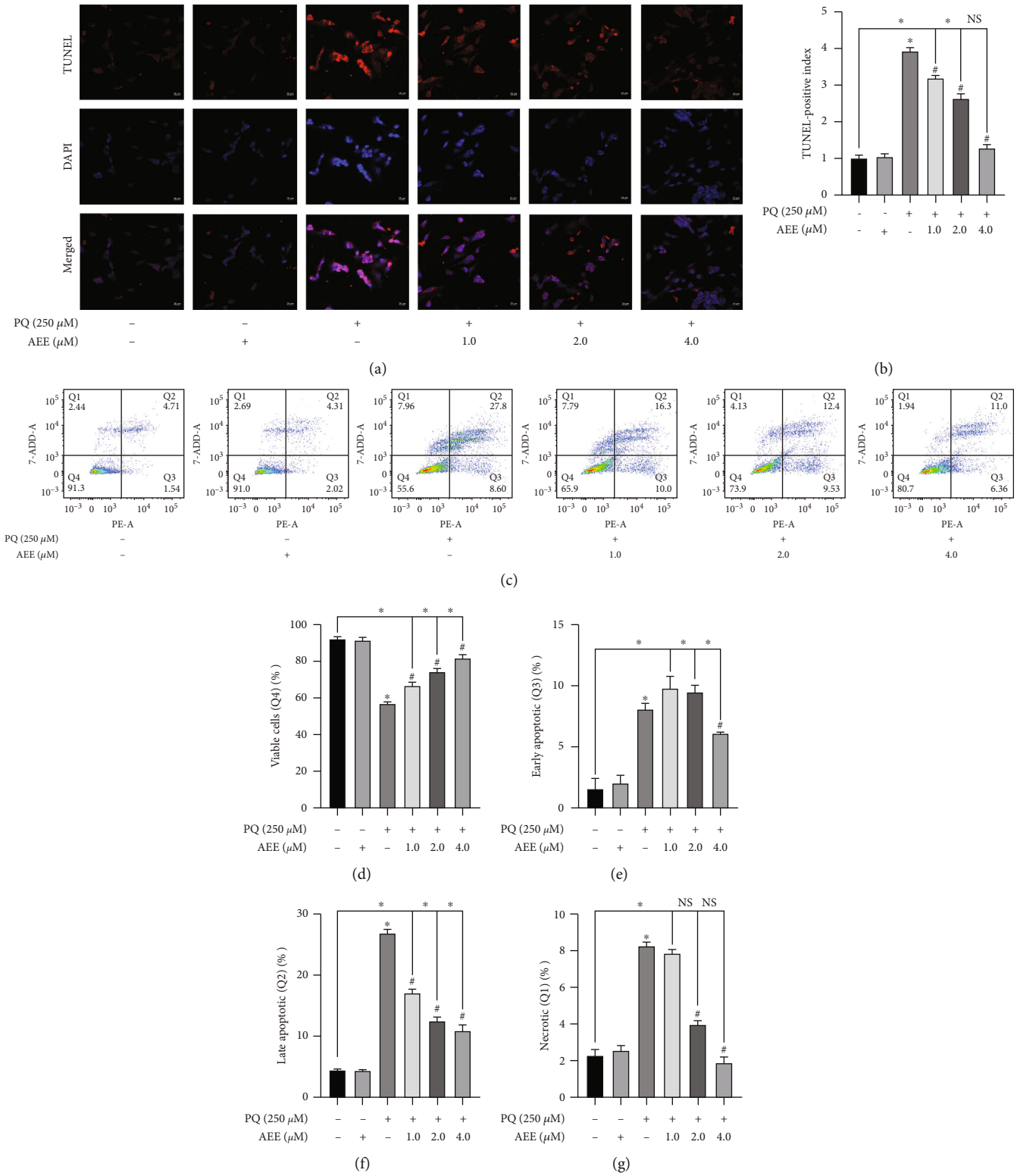
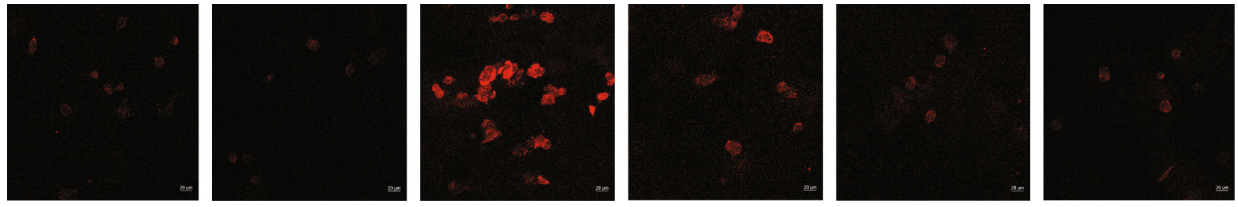
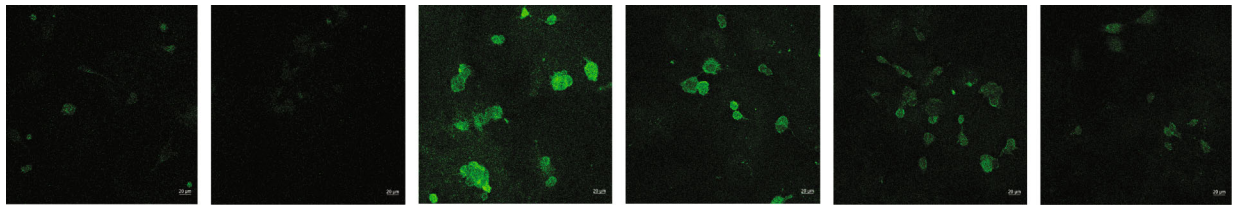


Figure 2: Effects of AEE on apoptosis in PQ-induced SH-SY5Y cells. (a, b) AEE significantly inhibited PQ-induced apoptosis of SY5Y cells by DAPI and TUNEL staining. Scale bar = 20 nm. Values are presented as the means ± SD where applicable ($n = 6$). (c–g) AEE significantly inhibited PQ-induced apoptosis of SH-SY5Y cells by flow cytometry. Values are presented as the means ± SD where applicable ($n = 3$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells.



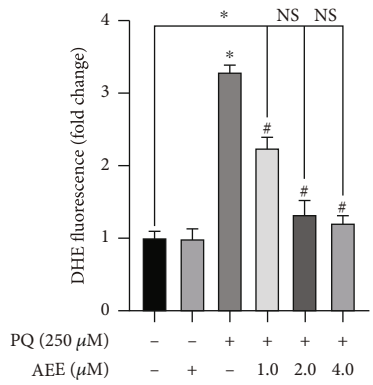
PQ (250 μM)	–	–	+	+	+	+
AEE (μM)	–	4.0	–	1.0	2.0	4.0

(a)

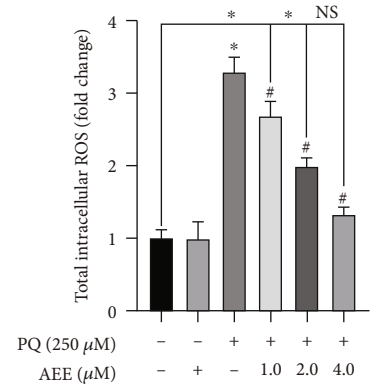


PQ (250 μM)	–	–	+	+	+	+
AEE (μM)	–	4.0	–	1.0	2.0	4.0

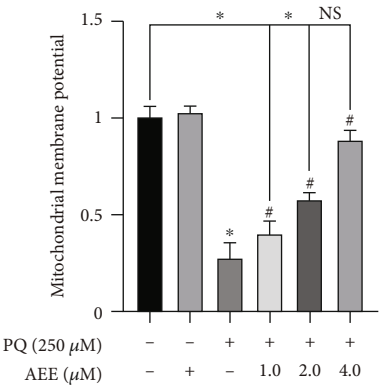
(b)



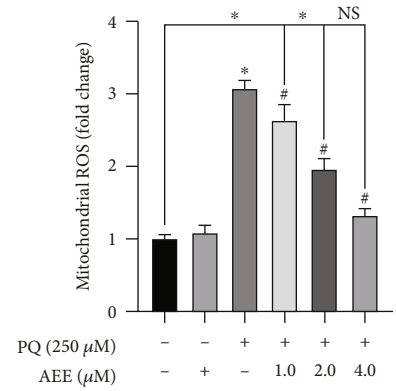
(c)



(d)

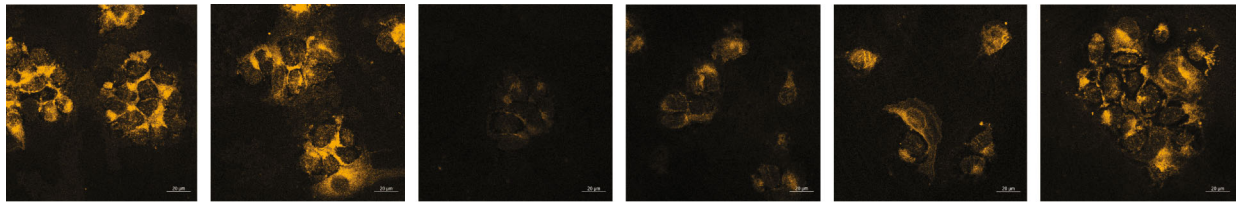


(e)



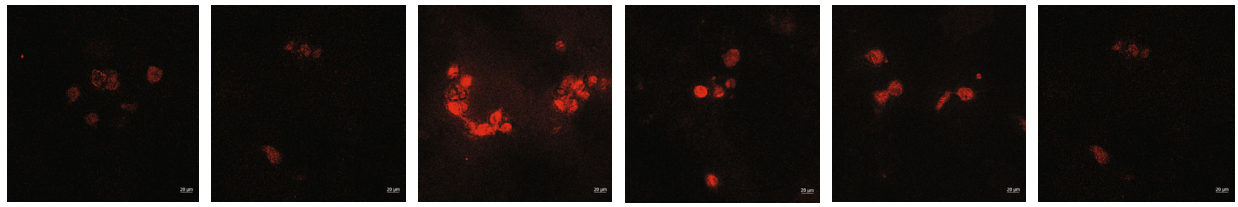
(f)

Figure 3: Continued.



PQ (250 μM)	–	–	+	+	+	+
AEE (μM)	–	4.0	–	1.0	2.0	4.0

(g)



PQ (250 μM)	–	–	+	+	+	+
AEE (μM)	–	4.0	–	1.0	2.0	4.0

(h)

Figure 3: AEE antagonizes PQ-induced oxidative stress in SH-SY5Y cells. (a, c) AEE significantly inhibited the increase of superoxide anion level in SH-SY5Y cells induced by PQ. (b, d) AEE significantly inhibited the increase of ROS level in SH-SY5Y cells induced by PQ. (e, g) AEE significantly inhibited the decrease of mitochondrial membrane potential induced by PQ in SH-SY5Y cells. (f, h) AEE significantly inhibited the increase of mitochondrial ROS in SH-SY5Y cells induced by PQ. Values are presented as the means ± SD where applicable ($n = 6$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells.

further experiments. Compared with the PQ group, the lower concentrations of AEE (0.5, 1, and 2 μM) only partially improved viability, and the concentration of 4 μM AEE was the most effective (Figure 1(c)).

3.2. AEE Inhibits PQ-Induced Apoptosis in SH-SY5Y Cells. The apoptosis morphology was evaluated by DAPI staining, and TUNEL assay was performed to assess apoptosis-induced DNA fragmentation. Compared with the control group, the number of TUNEL-positive cells (red fluorescence) in SH-SY5Y cells treated with PQ increased significantly. Compared with the control group, there was no significant difference in the number of TUNEL-positive cells (red fluorescence) in the 4.0 μM AEE+PQ treatment group (Figures 2(a) and 2(b)). The results of Annexin V-PE/7-AAD flow cytometry showed that PQ could significantly inhibit the survival of SH-SY5Y cells, while AEE could reduce the apoptosis of SH-SY5Y cells induced by PQ (Figures 2(c) and 2(d)). In addition, PQ could increase early apoptosis (Q3), late apoptosis (Q2), and necrosis (Q1) of SH-SY5Y cells, while 4 μM AEE could significantly attenuate the above phenomenon (Figures 2(e)–2(g)). The above results showed that AEE could inhibit the apoptosis of SH-SY5Y cells induced by PQ with an obvious concentration-dependent manner.

3.3. AEE Attenuates PQ-Induced Oxidative Stress in SH-SY5Y Cells. To verify the changes of redox status of SH-SY5Y cells, the levels of superoxide anion, intracellular ROS, and mitochondrial reactive oxygen species (mtROS) were detected. Analysis involved determination of pixels assigned to each cell using ImageJ software. Three images in each group were analyzed and each image analyzed 6 cells. AEE alone did not change the levels of superoxide anion, intracellular ROS, and mtROS. Compared with the control group, PQ could significantly increase the levels of superoxide anion, intracellular ROS, and mtROS. However, (4.0 μM) AEE pretreatment significantly inhibited PQ-induced superoxide anion, intracellular ROS, and mtROS levels in SH-SY5Y cells (Figures 3(a)–3(d), 3(f), and 3(h)). In addition, the mitochondrial membrane potential ($\Delta\Psi m$) was further detected. The results showed that AEE alone did not affect $\Delta\Psi m$ of SH-SY5Y cells (Figures 3(e) and 3(g)), compared with the control group. AEE pretreatment could significantly increase $\Delta\Psi m$ in SH-SY5Y cells, compared with the PQ group (Figures 3(e) and 3(g)). Compared with the control group, there was no significant difference in the 4.0 μM AEE+PQ treatment group. The results showed that AEE could significantly alleviate the mitochondrial dysfunction of SH-SY5Y cells via inhibiting intracellular ROS, mtROS, and superoxide anion levels.

3.4. AEE Enhances the Activities of ROS Scavenging Enzymes in PQ-Stimulated SH-SY5Y Cells. The activities of SOD, GSH-Px, CAT, and MDA were detected to explore the protective effect of AEE on SH-SY5Y cell induced by PQ. PQ could significantly increase the activity of MDA and decrease the activity of SOD, GSH-Px, and CAT, compared with the control group. However, AEE pretreatment could significantly increase the activities of SOD, GSH-Px, and CAT and decrease the activity of MDA (Figures 4(a)–4(d)). These results suggested that AEE pretreatment may attenuate PQ-induced oxidative damage in SH-SY5Y cells via increasing

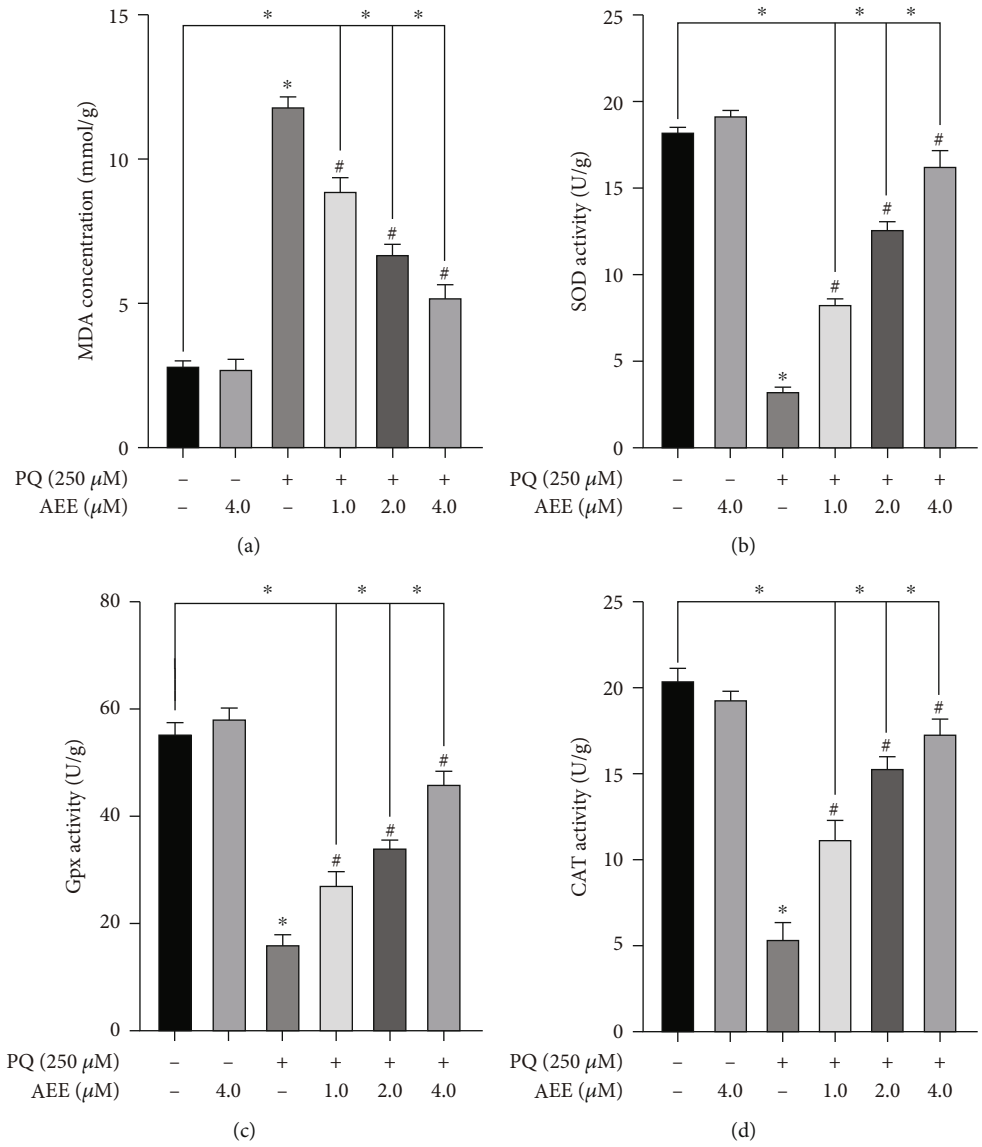


Figure 4: AEE enhances the activities of ROS scavenging enzymes in PQ-induced SH-SY5Y cells. (a) AEE significantly inhibited the increase of MDA level in SH-SY5Y cells induced by PQ. (b) AEE significantly inhibited the decrease of SOD level in SH-SY5Y cells induced by PQ. (c) AEE significantly inhibited the decrease of GSH-Px level in SH-SY5Y cells induced by PQ. (d) AEE significantly inhibited the decrease of CAT level in SH-SY5Y cells induced by PQ. Values are presented as the means ± SD where applicable ($n = 6$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "−": without the treatments in the SH-SY5Y cells.

the activity of ROS scavenging enzymes and inhibiting the activity of MDA.

3.5. AEE Regulates the Expression of Apoptosis-Related Proteins in SH-SY5Y Cells Induced by PQ. To further explore the molecular mechanism of AEE attenuating PQ-induced apoptosis in SH-SY5Y cells, the western blotting was used to detect the expression of apoptotic proteins (caspase-3, Bcl-2, and Bax). As shown in Figures 5(a)–5(d), compared with the control group, PQ could significantly inhibit the expression of Bcl-2 and significantly promote the expression of Bax and caspase-3. However, AEE pretreatment could significantly reverse the above changes. Compared with the control group, the ratio of Bcl-2/Bax and the expression of caspase-3 were not significantly different in the 4.0 μM AEE+PQ treatment group.

3.6. AEE Regulates the PI3K/Akt Signaling Pathways in SH-SY5Y Cells. Compared with the control group, the expression of p-Akt and p-PI3K in the PQ group decreased significantly. However, compared with the PQ group, AEE pretreatment could significantly upregulate the expression of p-Akt and p-PI3K in SH-SY5Y cells (Figures 6(a) and 6(b)). AEE pretreatment had no significant effect on the expression of Akt

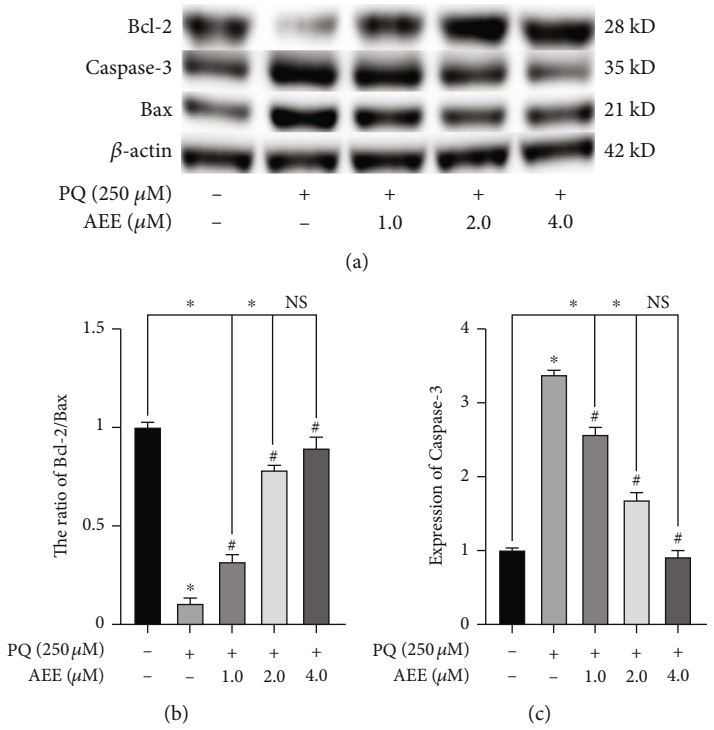


Figure 5: AEE regulates the expression of Bcl-2, Bax, and caspase-3 in SH-SY5Y cells induced by PQ. (a, b) AEE could significantly inhibit the decrease of Bcl-2/Bax ratio induced by PQ in SH-SY5Y cells. (a, c) AEE significantly inhibited the increase of caspase-3 protein in SH-SY5Y cells induced by PQ. Load in each lane was 40 μg protein. Values are presented as the means ± SD where applicable ($n = 3$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells.

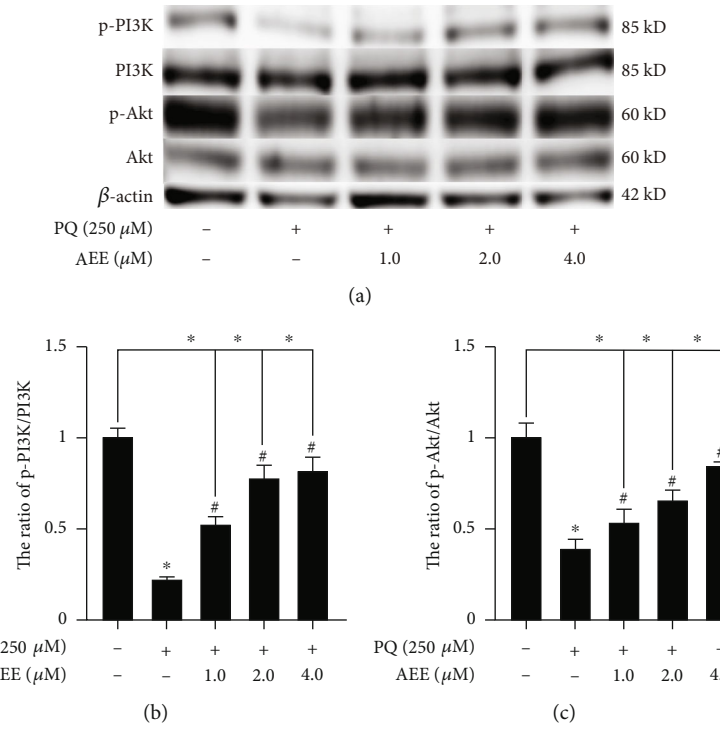


Figure 6: AEE regulates the expression of p-PI3K, PI3K, p-Akt, and Akt in SH-SY5Y cells induced by PQ. (a–c) AEE could increase the ratio of p-PI3K/PI3K and the ratio of p-AKT/Akt in SH-SY5Y cells induced by PQ. Values are presented as the means ± SD where applicable ($n = 3$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells.

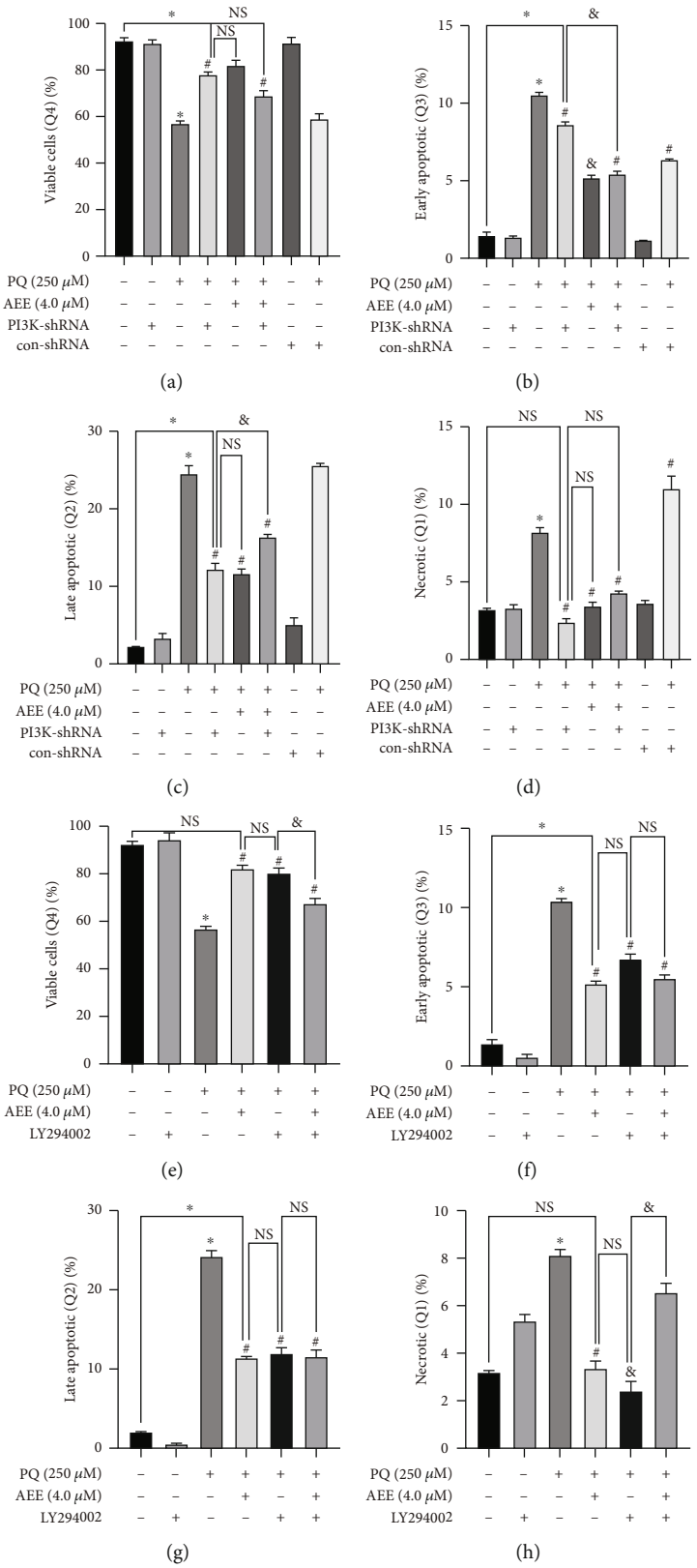


Figure 7: Continued.

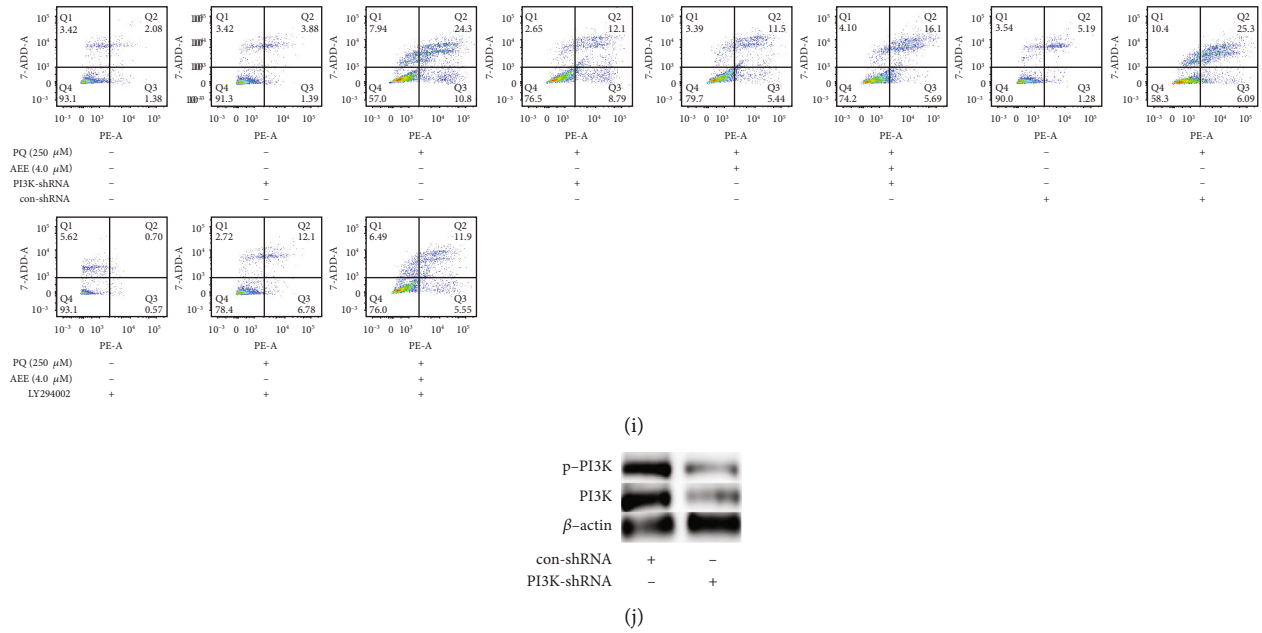


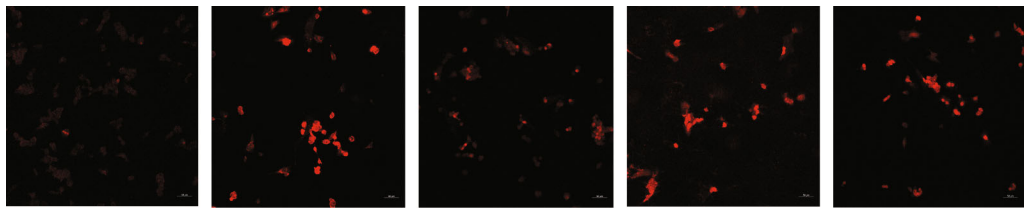
Figure 7: The effect of AEE on PQ-induced apoptosis after PI3K intervention with inhibitors and shRNA. (a–i) The effect of AEE on PQ-induced apoptosis after PI3K intervention with inhibitors and shRNA by flow cytometry. (j) The expression of p-PI3K and PI3K in the control-shRNA treatment groups and PI3K-shRNA treatment groups. Values are presented as the means ± SD where applicable ($n = 3$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells, $^{\&}p < 0.05$ compared with the shRNA PI3K+AEE+PQ group or LY294002+AEE+PQ group.

and PI3K in SH-SY5Y cells induced by PQ. These results suggested that AEE may possess protective potentials on PQ-induced SH-SY5Y cells via the PI3K/Akt pathway.

To further explore whether the PI3K/Akt pathway is the key pathway for AEE to protect SH-SY5Y cells, PI3K inhibitors LY294002 (10 μM) and shRNA were used to inhibit the expression of the PI3K/Akt signal pathway (Figure 7(g)). The results of flow cytometry showed that compared with the control group, the survival rate of SH-SY5Y cells in the PQ group was significantly reduced. PQ could increase early apoptosis (Q3), late apoptosis (Q2), and necrosis (Q1) of SH-SY5Y cells, while 4 μM AEE could significantly attenuate the above phenomenon (Figures 7(a)–7(d) and 7(i)). We have reinterpreted the data for Figures 7(a)–7(d) (populations of cells Q1, Q2, Q3, and Q4).

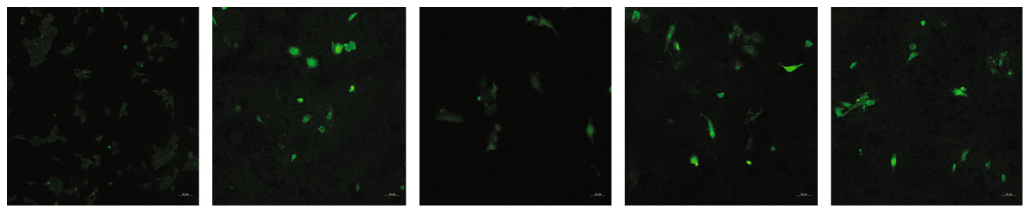
Compared with the PQ+AEE group, there was no significant difference in cell survival rate, late apoptotic cells, and necrotic cells in the shRNA PI3K+PQ or LY294002+PQ group (Figures 7(a), 7(c), 7(d), 7(e), 7(g), 7(h), and 7(i)). Interestingly, compared with the PQ+AEE group, there were significant differences in early apoptotic cells in the shRNA PI3K+PQ group (Figure 7(b)). On the contrary, compared with the PQ+AEE group, there was no significant difference in early apoptotic cells in the LY294002+PQ group (Figure 7(f)). The results showed that the effect of inhibition of PI3K (by shRNA PI3K or LY294002) was protective in similar way, compared with the PQ+AEE group. However, the shRNA PI3K+PQ group was not statistically different from the PQ+AEE group, although there was some tendency in weakening by the effect of AEE in population of late apoptotic cells (Figure 7(c)). Compared with the shRNA control+PQ group, there was no significant difference in cell survival rate and necrosis in the shRNA PI3K+AEE+PQ group, while there were significant differences between early apoptotic cells and late apoptotic cells. It could be observed that the population of late apoptotic cells (Figure 7(c)) in the shRNA control+PQ group was in as much as the PQ group and much higher number of cells in the shRNA control+PQ than the PQ group in Figure 7(d). However, compared with the AEE+PQ group, there was no significant difference in cell survival rate, early apoptotic cells, and late apoptotic cells in the LY294002+PQ group, while there were significant differences in necrotic cells (Figures 7(e)–7(i)). From Figure 7(h), LY294002 inhibitor of PI3K seemed to reverse the effect of AEE on PQ, but it was protective when given with PQ (Q1, necrotic cells). This may result from mechanical injury in test operation because LY294002 can inhibit cell apoptosis if all apoptosis cells in Q1, Q2, and Q3 were incorporated to analyze. When LY294002 inhibitor is used, the protective effect of AEE on SH-SY5Y cells was significantly weakened, which showed there were some antagonisms between LY294002 and AEE.

Compared with the PQ group, AEE pretreatment significantly inhibited PQ-induced superoxide anion, intracellular ROS, and the number of TUNEL-positive cells and significantly improved $\Delta\Psi m$ in SH-SY5Y cells (Figures 8(a)–8(h)). Compared with the PQ group, the levels of superoxide anion, intracellular ROS, $\Delta\Psi m$, and the number of TUNEL-positive cells were not significantly different in the LY294002+PQ treatment group or the PI3K shRNA+PQ treatment group. Western blot results showed that the ratio of Bcl-2 and Bax and the expression of caspase-3 were not



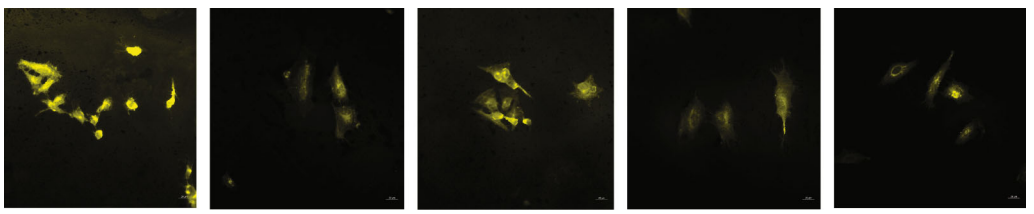
PQ (250 μM)	−	+	+	+	+
AEE (4.0 μM)	−	−	+	+	+
PI3K-shRNA	−	−	−	+	−
LY294002	−	−	−	−	+

(a)



PQ (250 μM)	−	+	+	+	+
AEE (4.0 μM)	−	−	+	+	+
PI3K-shRNA	−	−	−	+	−
LY294002	−	−	−	−	+

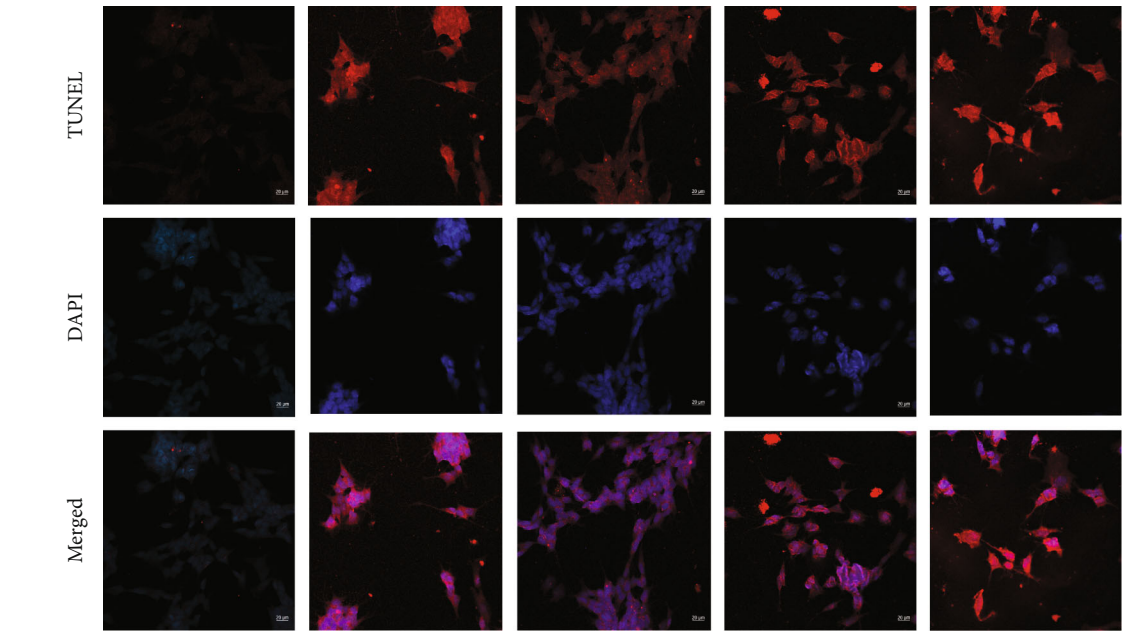
(b)



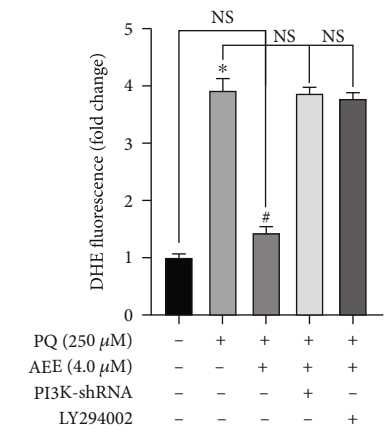
PQ (250 μM)	−	+	+	+	+
AEE (4.0 μM)	−	−	+	+	+
PI3K-shRNA	−	−	−	+	−
LY294002	−	−	−	−	+

(c)

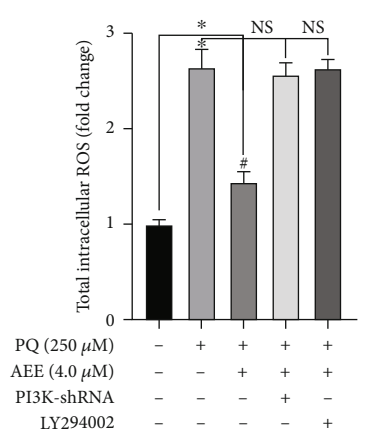
Figure 8: Continued.



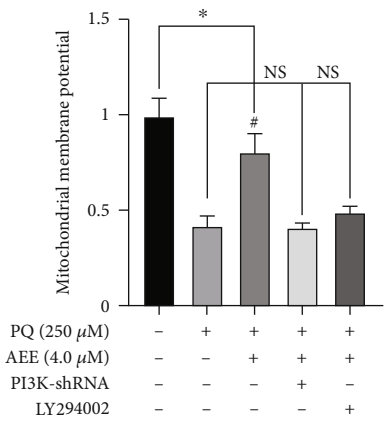
(d)



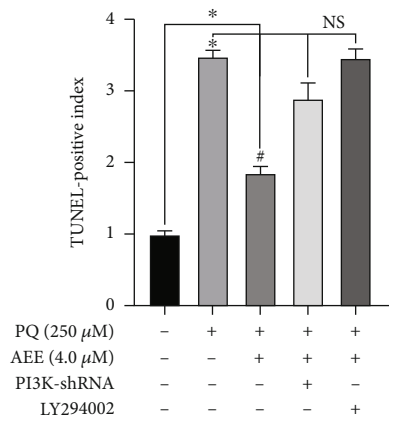
(e)



(f)



(g)



(h)

Figure 8: Continued.

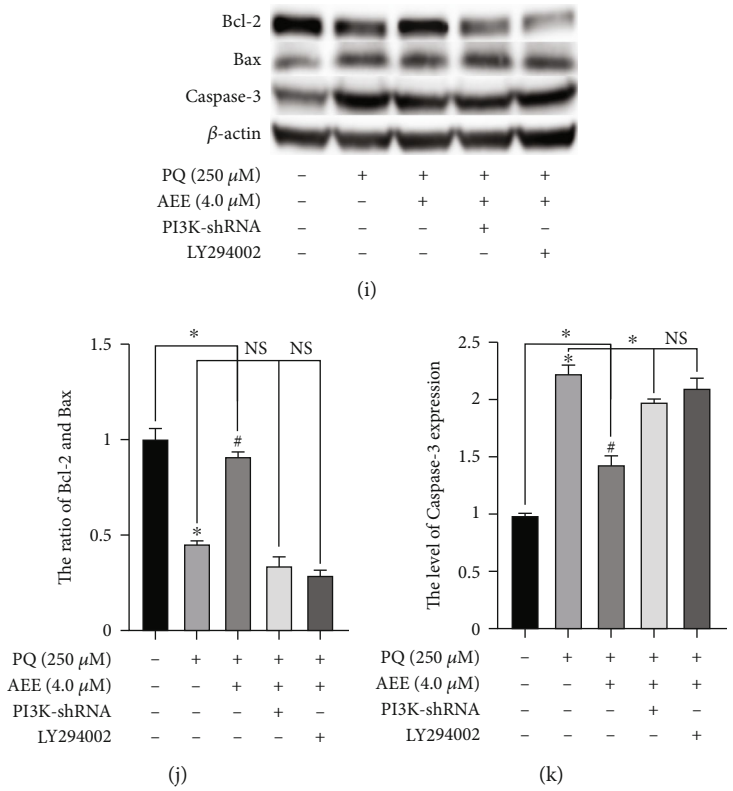


Figure 8: Intervention of PI3K with inhibitors and shRNA could weaken the protective effect of AEE. (a, e) Intervention of PI3K with inhibitors and shRNA could weaken the inhibitory effect of AEE on DHE. Scale bar = 50 nm. (b, f) Intervention of PI3K with inhibitors and shRNA could weaken the inhibitory effect of AEE on ROS. Scale bar = 50 nm. (c, g) Intervention of PI3K with inhibitors and shRNA could weaken the effect of AEE on the improvement of mitochondrial membrane potential. Scale bar = 20 nm. (d, h) Intervention of PI3K with inhibitors and shRNA could weaken the protective effect of AEE on cell apoptosis via DAPI and TUNEL staining. Scale bar = 20 nm. (i–k) Intervention of PI3K with inhibitors and shRNA could weaken the protective effect of AEE on cell apoptosis via western blot. Values are presented as the means ± SD where applicable ($n = 6$). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the PQ group, and no significant difference (NS) $p > 0.05$. "+": with the treatments in the SH-SY5Y cells; "–": without the treatments in the SH-SY5Y cells.

significantly different in the LY294002+PQ treatment group or the PI3K shRNA+PQ treatment group (Figures 8(i)–8(k)). The above results showed that inhibition of PI3K will weaken AEE-mediated protection.

4. Discussion

As we all know, the pathogenesis of PD is mediated by excessive production of ROS, which will eventually lead to the loss of mitochondrial membrane potential, resulting in the activation of caspase cascade [38–41]. In this study, PQ induced oxidative stress in SH-SY5Y cells, and the production of ROS was the initial event that mediated the death of SH-SY5Y cells. The present study revealed that AEE pretreatment significantly reduced the excessive production of intracellular ROS and mtROS, which may be one of the reasons for AEE inhibiting PQ-induced apoptosis in SH-SY5Y cells. Based on further study, cell stimulation by external factors will increase the level of Bax, and Bax promotes the release of cytochrome c from the mitochondria to the cytoplasm, thus inducing neuronal apoptosis [42–44]. PQ could significantly increase the expression of Bax, and AEE pretreatment could attenuate the expression of Bax induced by PQ in a concentration-dependent manner.

Mitochondrial dysfunction is considered a critical mechanism underlying the pathogenesis of PD. PQ promoted oxidative stress at mitochondrial level, which, in turn, impacted on the morphology of these organelles and, ultimately, on cell viability [45]. Studies showed that PQ could induce the formation of free radicals, mitochondrial dysfunction, mitosis, and activation of PINK1 protein in SY5Y cells. Lee et al. could attenuate PQ-induced neurotoxicity by mediating mitochondrial dysfunction and mitosis [46]. Ju et al. reported that PQ induces the accumulation of double-membrane autophagic vacuoles (AVs) in the cytoplasm of SH-SY5Y cells. PQ enhanced ROS-mediated neuroinflammation, oxidative stress, and apoptosis in SH-SY5Y cells. However, the effect of PQ was counteracted by vasicinone treatment, which activated the IGF-1R/AKT/PI3K signaling pathway to inhibit MAP kinases and the expression of apoptotic proteins such as Bax and Bad, inhibited cytochrome c release, and inhibited the cleavage of caspase-9, caspase-3, and PARP, suppressing cell death [47]. PQ also could induce mitochondrial dysfunction through NRF2 transcription factors and miR-34a and increase the expression of Bcl-2 family proteins and BDNF

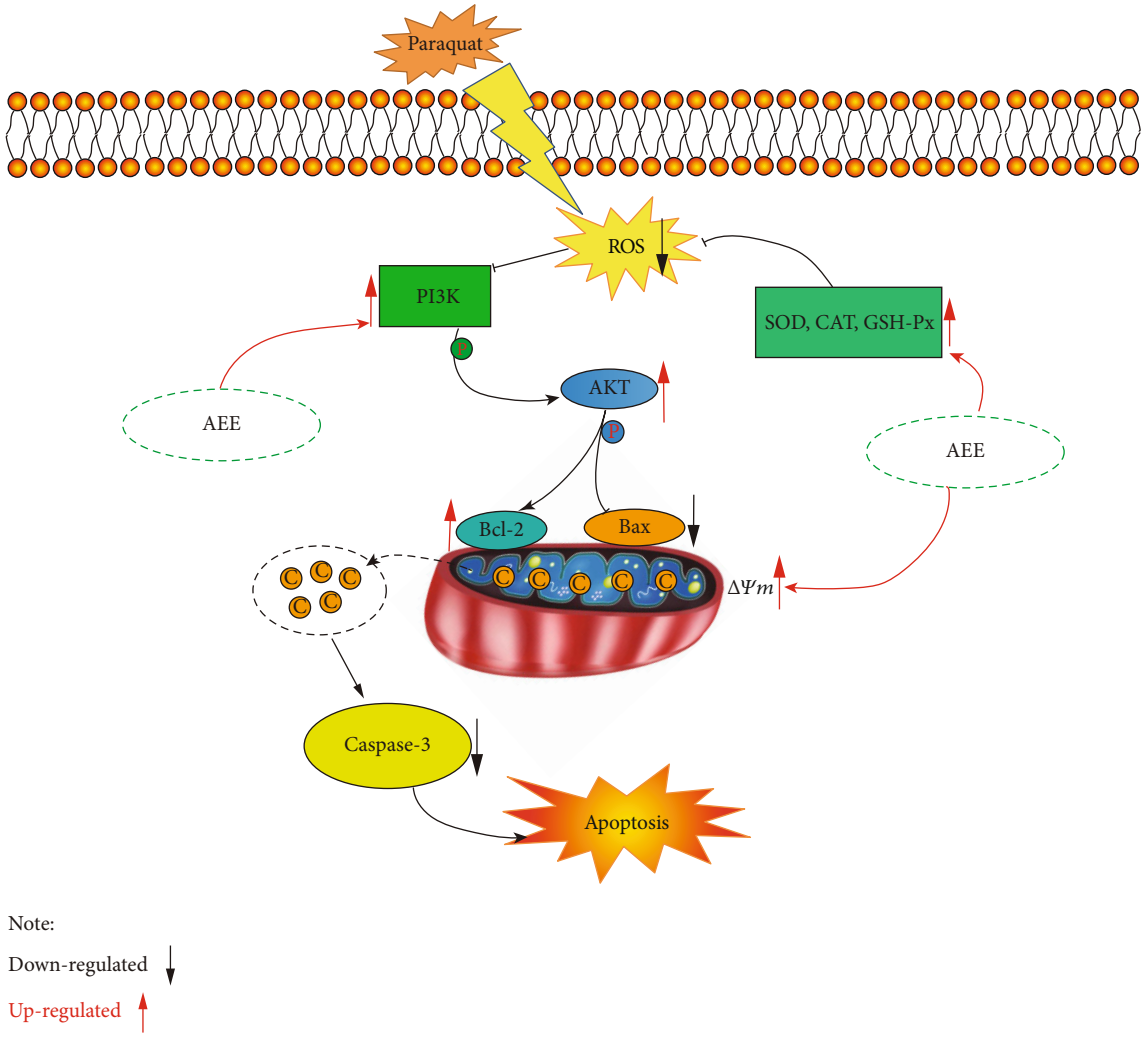


Figure 9: The molecular mechanism of AEE inhibiting PQ-induced apoptosis in SH-SY5Y cells.

mRNA [48]. However, some studies showed that PQ-induced apoptosis of SH-SY5Y cells is independent of mitochondrial dysfunction [49]. PQ could directly participate in the oxidative cycle by increasing the level of ROS and lead to caspase-independent cell death, which is similar to programmed cell necrosis.

The important characteristics of apoptosis are caspase cascade activation, DNA fragmentation, and nuclear pyknosis [50, 51]. Bax exhibits proapoptotic actions, whereas Bcl-2 has an antiapoptotic effect [52]. The delicate balance between Bax and Bcl-2 regulates cell integrity and controls cell survival. When this balance is broken by external factors, it activates the signaling process of cell death [53]. In mammalian cells, Bcl-2 family proteins regulate the release of mitochondrial cytochrome c into the cytoplasm and further activate caspase family proteins [54, 55]. The studies showed that AEE protects nerve cells from oxidative stress and apoptosis induced by PQ, which is proved by the decrease of Bax protein level and the increase of Bcl-2 protein level.

Our previous studies have shown that AEE treatment significantly reduced H_2O_2-induced oxidative stress in HUVECs via mitochondria-lysosome axis and Bcl2 was an important regulation target of AEE to protect cells from oxidative stress [28]. AEE decreased lipid peroxidation and enhanced antioxidant ability in the HUVECs and mitigated mitochondrial dysfunction induced by H_2O_2. Similar to this study, AEE could also improve the mitochondrial dysfunction of SH-SY5Y cells induced by PQ via inhibiting oxidative stress. Different from the previous studies, AEE attenuated PQ-induced apoptosis in SH-SY5Y cells via the PI3K/AKT signal pathway.

The PI3K/Akt signaling pathway plays an important role in cell survival, differentiation, proliferation, and apoptosis [56–58]. Phosphatidylinositol 3 kinase (PI3Ks) belongs to the lipid kinase family, which phosphorylates inositol phosphate at the D-3 position of the inositol head group, resulting in the production of the D-3 phosphate. PI3K mediates extracellular signal transduction and regulates a variety of cellular events, including cell mitosis, cell survival, and membrane transport. According to the enzyme domain structure and substrate specificity of PI3K, it can be divided into three categories in mammals (I-III). Among them, the class I subfamily is the most widely studied. The class I subfamily consists of four catalytic subunits, including three IA

subunits (p110-α, p110-β, and p110-δ) and one IB subunit (p110-γ). When phosphorylation of PI3K increases, it transduces signals through inositol 3-phosphate-dependent protein kinase-1 (PDK1), a serine/threonine kinase. PDK1 is recruited to the cell membrane after PI3K activation, where it phosphorylates and activates Akt, the main medium of the PI3K signal transduction pathway. Akt, a serine/threonine kinase, is pivotal in cellular metabolism, growth, and survival [59, 60]. When Akt is activated, it plays a key role in PI3K-mediated signal transduction [61–63]. The phosphorylation of AKT can increase the expression of Bcl-2 and inhibit the expression of Bax in the mitochondria. LY294002 is not only a competitive DNA-PK inhibitor but also a commonly used PI3K drug inhibitor, which acts on the ATP binding site of PI3K enzyme, thus selectively inhibiting PI3K-Akt connection. Pretreatment with LY294002 for 2 h significantly counteracted the protective effect of AEE. Consistent with this, using shRNA to knock down PI3K has a similar result (Figure 8). PQ treatment of SH-SY5Y cells resulted in excessive production of intracellular ROS. The phosphorylation of PI3K can be inhibited by excessive production of ROS. However, AEE pretreatment could inhibit the decrease of PI3K phosphorylation induced by PQ. With the recovery of mitochondrial membrane potential, the mitochondria will reduce the release of cytochrome c and inhibit the activation of caspase family. At the same time, the enzyme activities of CAT, SOD, and GSH-Px were changed by AEE pretreatment, which further eliminated the excess ROS in the SH-SY5Y cells. Both LY294002 and shRNA could inhibit the expression of PI3K. When SH-SY5Y cells were interfered by inhibitors LY294002 and shRNA, compared with the LY294002 group, the inhibitory effect of the AEE+LY294002 group on PQ-induced apoptosis of SH-SY5Y cells was weakened. Similarly, compared with the PI3K shRNA group, the inhibitory effect of the AEE+-PI3K shRNA group on PQ-induced apoptosis of SH-SY5Y cells was also weakened. The results showed that AEE can alleviate PQ-induced apoptosis of SH-SY5Y cells via upregulating the expression of p-PI3K, p-Akt, and Bcl-2 and downregulating the expression of caspase-3 and Bax (Figure 9).

5. Conclusion

PQ enhanced the oxidative stress and apoptosis of SH-SY5Y cells mediated by ROS. AEE pretreatment inhibited cell death by activating the PI3K/Akt signal pathway, inhibiting the expression of apoptotic proteins such as Bax and Bad, and inhibiting the cleavage of caspase-3. There is reasonable evidence to support that AEE may be a new potential drug to treat neurodegenerative diseases for further *in vivo* studies.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

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Review Article

Procaine–The Controversial Geroprotector Candidate: New Insights Regarding Its Molecular and Cellular Effects

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Since its discovery in 1905 and its employment in everyday medical practice as a local anesthetic, to its highly controversial endorsement as an "anti-aging" molecule in the sixties and seventies, procaine is part of the history of medicine and gerontoprophylaxis. Procaine can be considered a "veteran" drug due to its long-time use in clinical practice, but is also a molecule which continues to incite interest, revealing new biological and pharmacological effects within novel experimental approaches. Therefore, this review is aimed at exploring and systematizing recent data on the biochemical, cellular, and molecular mechanisms involved in the antioxidant and potential geroprotective effects of procaine, focusing on the following aspects: (1) the research state-of-the-art, through an objective examination of scientific literature within the last 30 years, describing the positive, as well as the negative reports; (2) the experimental data supporting the beneficial effects of procaine in preventing or alleviating age-related pathology; and (3) the multifactorial pathways procaine impacts oxidative stress, inflammation, atherogenesis, cerebral age-related pathology, DNA damage, and methylation. According to reviewed data, procaine displayed antioxidant and cytoprotective actions in experimental models of myocardial ischemia/reperfusion injury, lipoprotein oxidation, endothelial-dependent vasorelaxation, inflammation, sepsis, intoxication, ionizing irradiation, cancer, and neurodegeneration. This analysis painted a complex pharmacological profile of procaine: a molecule that has not yet fully expressed its therapeutic potential in the treatment and prevention of aging-associated diseases. The numerous recent reports found demonstrate the rising interest in researching the multiple actions of procaine regulating key processes involved in cellular senescence. Its beneficial effects on cell/tissue functions and metabolism could designate procaine as a valuable candidate for the well-established Geroprotectors database.

1. Introduction

Procaine was synthesized by Alfred Einhorn in 1905 and introduced in clinical practice as Novocain, soon becoming a local anesthetic prototype. Around the 1950s, a large number of accumulated data emphasized the surprising diversity of nonanesthetic effects exerted by procaine, which came to the attention of various medical research schools in Eastern and Western Europe, many doctors exploring, regardless of borders, the beneficial properties of procaine: Vishnevsky and Speransky (Russia); Huneke and Lüth (Germany); Leriche, Dos Ghali, and Hazard (France); Danielopolu and Parhon (Romania) [1–9]. Between 1946 and 1956, Ana Aslan described a significant number of procaine beneficial actions exerted on cellular functions and metabolism, following long-term treatment in low doses, highlighting its "rejuvenating" effects, and developed Gerovital H3 (GH3)—an original procaine-based pharmaceutical formulation [10–12]. Due to

these findings, procaine which was known only for its anesthetic properties became one of the most disputed medical developments of the sixties and seventies in the field of "anti-aging" therapy [13–15].

While aging *per se* seems to be the predominant risk factor for most diseases that limit healthspan, the human lifespan can be viewed as a series of gene-environment interactions that inevitably lead to an earlier or later onset of aging-related conditions such as type 2 diabetes, atherosclerosis and cardiovascular diseases, depression, neurodegeneration and cognitive decline, cancer, sarcopenia, osteoarthritis, and osteoporosis [16, 17].

Recent progresses in the field of aging research led to the development of a new class of drugs—geroprotectors, with the ability to target fundamental mechanisms of aging common to multiple age-related diseases, such as response to oxidative damage, inflammation, hypermethylation, cellular senescence, and autophagy [18]. Moskalev et al. (2015) established the first public database of geroprotectors (http://geroprotectors.org) that indexes the most relevant experiments involving over 200 well-established geroprotectors or possible candidates that could extend the healthy lifespan and repair or reduce aging-related damage in model organisms [19]. As primary selection criteria for the potential geroprotectors, the following characteristics were recognized: (1) the ability to increase lifespan; (2) the capacity to ameliorate molecular, cellular, and physiological biomarkers to a younger state or slow the progression of age-related change of these markers; (3) a therapeutic lifespan-extending dose of geroprotector, which should be several orders of magnitude less than the toxic dose; and (4) the capacity to improve the health-related quality of life of the patient, from a physical, mental, emotional, and social viewpoint [20]. The compliance of procaine with most of these criteria would allow it to be a potential "geroprotector" candidate.

Although GH3 was internationally launched in 1956, simultaneously with the development of the Free Radical Theory of Aging by Denham Harman [21], the study of the antioxidant action of procaine and GH3 was documented only after 1980, in various experimental designs, which proved its capacity of limiting the generation of reactive oxygen species (ROS) and lipid peroxidation [22–29]. Recently, the radioprotective effects of procaine and GH3 were reported *in vitro* in human lymphocytes isolated from young and aged individuals [29]. Besides its antioxidant, cytoprotective, anti-inflammatory, and antiatherogenic effects, at cellular and molecular levels, procaine has multiple targets, supporting a large number of potential "geroprotective" effects [30, 31]. Older and more recent data revealed that procaine and its metabolites modulate several biochemical and cellular processes like mitochondrial structure and function [32–34], cholesterol biosynthesis [35], monoamine oxidase (MAO) activity [36, 37], and DNA methylation [38–41].

Procaine is part of the history of medicine and gerontoprophylaxis, an old-timer of clinical practice, but still a molecule with great potential which continues to reveal new biological and pharmacological effects within novel experimental approaches. Therefore, the aim of this review is to explore and systematize data on biochemical, cellular, and molecular mechanisms involved in the antioxidant and alleged geroprotective actions of procaine and GH3, focusing on the following aspects: (1) the research state-of-the-art, through an objective examination of scientific literature for the last 30 years—describing both positive and negative research outcomes; (2) the experimental data supporting the beneficial effects of procaine in preventing the age-related pathology; and (3) the multitude of ways procaine impacts oxidative stress, atherogenesis, cerebral age-related pathology, and DNA methylation.

2. Procaine and Gerovital H3—From Anesthetic to "Anti-Aging"

As early as 1892, the German chemist Alfred Einhorn began to model on the structural formula of cocaine, an alkaloid extracted from the leaves of *Erythroxylum coca* and the first known local anesthetic, in order to obtain less addictive molecules, but with similar or enhanced anesthetic qualities. Thus, he synthesized procaine—the first injectable anesthetic, introduced in medical practice under the trade name of *Novocaine*, which means "new cocaine," from the Latin *nov-* "new" and *-caine*, a common ending for alkaloids used as anesthetics [42].

Therapeutic effects pointed out after systemic administration and anesthetic properties, illustrate different aspects of procaine pharmacodynamics, being determined by dosage and administration routes. It was known by then that the systemic administration of procaine at high concentrations leads to a local anesthetic effect. Of all the local anesthetics, procaine was the least toxic [43–45]. In 1949, the physiologists Danielopolu and Simionescu highlighted procaine's multiplicity of actions, stating that it "exerts uniform action in the organism, restores and enhances active vital processes and local resistance" [8]. Among the researchers who took a close look at procaine, Ana Aslan noticed another quality of procaine: its geroprotective properties. In 1951, she began treating a group of selected patients with 2% procaine, reporting several "rejuvenating" effects in elderly patients: memory enhancement, alleviation of depression, hair repigmentation, better skin tone, and an overall improvement of their condition [10, 11]; all these observations resulted, at that stage, in regarding procaine as "a useful prophylactic and therapeutic substance in the fight against old age" [9, 12]. At the same time, due to its short-term anesthetic effect and need for repeated administration to achieve longer anesthesia, observations regarding the interesting changes occurring following prolonged use started to emerge in the medical community [46]. Aslan sought to alter procaine pharmacokinetics in order to increase its stability in the body, prolong its action and explore its full therapeutic potential. In this purpose, in 1956, a pharmaceutical formulation containing 2% procaine hydrochloride, 0.12% benzoic acid, 0.10% potassium metabisulphite, and 0.01% disodium phosphate (as excipients and stabilizers), with a pH of 3.3, was designed and marketed as GH3 [12, 30]. Pharmacokinetic studies revealed that serum procaine levels are higher after the administration of GH3 than following the one of a procaine solution of similar concentration [47, 48]. Although the

studies regarding the effects of GH3 were developed within a large prophylaxis campaign and there were clinical trials involving thousands of elderly subjects, some in the medical world of the 1960s contradicted the so-claimed beneficial effects of the treatment developed by Aslan [13]. The negative outlook and backlash were caused because, at that time, behind Gerovital was more marketing for a "miraculous anti-aging product" than indisputable scientific evidence. In 1982, following the study commissioned by the National Institute on Aging, the U.S. Food and Drug Administration (FDA) banned GH3 for "anti-aging and associated claims" [13–15].

3. Procaine Pharmacokinetics and Metabolism

Procaine is a drug with limited distribution and tissue uptake and a short duration of action: during a continuous intravenous infusion of 2% procaine, the steady-state plasma level is achieved within 20 to 30 minutes. Following the termination of the administration, drug concentration decreases rapidly, with a distribution half-life (t1/2 alpha) of 2.49 ± 0.36 minutes and an elimination half-life (t1/2 beta) of 7.69 ± 0.99 minutes [49]. At systemic level, procaine is hydrolyzed to diethyl-amino-ethanol (DEAE) and *para*-aminobenzoic acid (PABA), as primary metabolites, by the enzyme pseudocholinesterase [50] (Figure 1). In different organs, procaine is hydrolyzed under the microsomal carboxylesterases [51, 52]. DEAE displays local anesthetic activity [53].

Procaine hydrolysis represents an important feature that could support some of its effects on cellular functions and metabolism, as the primary and secondary metabolites could have additional pharmacologic actions or participate as precursors in the synthesis of essential biomolecules. Kietzmann and Kaemmerer (1989) tested the influence of orally-administrated procaine hydrochloride on intermediary metabolism in rats and pointed out the fact that the ratio of acetyl coenzyme A to coenzyme A clearly was enhanced in the liver and, to a minor extent, in the cerebellum. Also, procaine hydrochloride, GH3, as well as DEAE increased, in a dose- and time-dependent mode, the hepatic incorporation rate of amino acids in protein, while PABA yielded no effect [54].

A very attractive hypothesis, which needs more studies and scientific evidence, is the second stage of procaine metabolism (remarkable from the pharmacodynamics viewpoint): the possible generation of ethanolamine from DEAE. Ethanolamine is a possible precursor in the biosynthesis of membrane phospholipids (phosphatidylethanolamine and phosphatidylcholine), which can be converted into the neurotransmitter acetylcholine (Ach) [55–57].

4. Aging, Age-Related Diseases, and Antioxidant Action of Procaine and GH3

The "Free Radical Hypothesis of Aging" was put forward 65 years ago, being later revised to the theory known as the "Oxidative Stress Hypothesis" [21, 58, 59]. According to these theories, oxidative stress is caused by the imbalance between the reactive oxygen species (ROS) production and the biological system's ability to counteract, with an appropriate antioxidant defense, resulting in the oxidative damage of cell membranes and other structures such as lipids, lipoproteins, proteins, and DNA [60]. Multiple endogenous sources such as xanthine oxidase, NADPH oxidase, and the mitochondrial respiratory chain can be involved in ROS generation. A variety of environmental stimuli, such as radiation, pathogen infections, and exposure to xenobiotics, can also enhance *in vivo* ROS production [59].

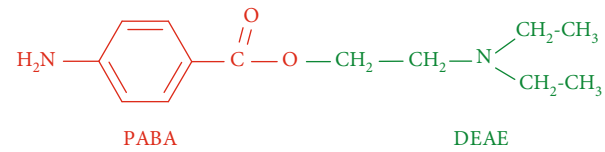

Figure 1: Chemical structure of procaine, ester of *para*-aminobenzoic acid (PABA) with diethyl-amino-ethanol (DEAE).

Since many age-related diseases/geriatric syndromes are associated with oxidative stress, and the consequent cellular damage, limiting its intensity became a major area of interest and a common therapeutic target of aging-related pharmaceutical research [61]. Among the strategies of disease prevention and geroprotective therapies, antioxidants are currently still of cutting-edge interest. New experimental approaches, along sensitive and specific methods, are employed in testing and evaluating the actions of natural antioxidant compounds or drugs on alleviating oxidative stress under biological conditions similar to those existing *in vivo* [62, 63].

The antioxidant action of procaine and GH3 has been supported by *in vitro* and *in vivo* studies, in different research models demonstrating the inhibition of ROS generation and lipid peroxidation, associated with a modulating effect on antioxidant enzymes and nonenzymatic antioxidants.

4.1. ROS Generation and Lipid Peroxidation—In Vitro Studies. In 1989, Rusu and Lupeanu demonstrated for the first time the antioxidant action of procaine, but also of the other GH3 ingredients, namely, potassium metabisulphite and benzoic acid. The inhibitory effect of these products on ROS generation was evaluated *in vitro* using Nishikimi's electron carrier system generating the superoxide radical ($O_2^{\cdot -}$), comprising reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), and nitroblue tetrazolium (NBT). GH3 and procaine inhibited the generation of $O_2^{\cdot -}$ in the presence of increasing concentrations of procaine hydrochloride equivalents (0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 mM), compared to Cu/Zn-superoxide dismutase (SOD)—the antioxidant enzyme that detoxifies superoxide physiologically. The strongest antioxidant effect (68% inhibition of NBT reduction) was exerted at 2.0 mM GH3 [22].

It was proposed that toxic O_2 metabolites generated by xanthine oxidase (XO) contribute *in vivo* to the development of ischemia-reperfusion injury in a variety of tissues [59]. Gradinaru et al. (2009) examined the antioxidant effects of procaine and GH3 with regard to $O_2^{\cdot -}$ generation in a physiologic enzymatic system: xanthine – XO – 2-(4-

iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT). GH3 inhibited INT reduction in a dose-dependent manner, at degrees comparable to SOD. At 10 mM, the maximum inhibition of $O_2^{\cdot -}$ generation was achieved by GH3 (62%), whereas procaine hydrochloride had a slight (5%) inhibitory effect [25].

Previously, Jinnouchi et al. (2005) studied whether local anesthetics inhibit the priming of neutrophils induced by lipopolysaccharide (LPS). They found that 4.0 mM procaine, 3.0 mM lidocaine, 0.5 mM bupivacaine, or 0.1 mM tetracaine inhibited by 50% the release of $O_2^{\cdot -}$, in response to triggering by the chemotactic peptide N-formyl-methionyl-leucyl phenylalanine (fMLP) [64]. Librowski and Moniczewski (2010) examined comparatively the antioxidant effect of several local anesthetics. The potency of scavenging radicals, measured as relative scavenging effect (%) of the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) cation, decreased in the following order: tetracaine > procaine > lignocaine > benzocaine (by 99, 38, 21, and 20%, respectively, at a concentration of 10 mM) [27].

Lee et al. (2010) tested *in vitro* the antioxidant effect of procaine and lidocaine on endothelial-dependent relaxation in the rabbit aorta to examine if their antioxidizing effects could suppress or reduce the ROS-induced endothelial damage. The isolated aortic rings were pretreated with procaine or lidocaine (10^{-5} M to 3×10^{-3} M) and subjected to precontraction with phenylephrine (PE). The changes (%) of the aortic tone by acetylcholine (ACh) administration before (control) and after ROS exposure were compared. Their results suggested that procaine and lidocaine dose-dependently preserved endothelium-dependent vasorelaxation against ROS attack, procaine potentially acting via hydrogen peroxide scavenging, as part of its protection mechanism [26]. Takaishi et al. (2013) evaluated the effects of local anesthetics procaine and lidocaine on nitric oxide (NO) production in a bovine aortic endothelial cells culture (BAEC), under proinflammatory conditions. In bradykinin and Ach stimulated cells, 10 mM procaine significantly inhibited NO production by 35%, whereas in cells incubated with interleukin-1 beta (IL-1β) and LPS, 10 mM procaine significantly inhibited NO production by 15%. Authors suggested that the inhibitory effects of procaine on NO production are partially due to the suppression of L-arginine uptake [65].

The antioxidant action of GH3 and procaine under proinflammatory conditions was recently tested by Ungurianu et al. (2020) using a human lymphoblastoid cell line as experimental model. Membrane lipid peroxidation in Jurkat cells was induced by cumene hydroperoxide (CuOOH) and assessed with diphenyl-1-pyrenylphosphine (DPPP), a sensitive fluorescent probe. The preincubation of Jurkat cells with 2.5, 5.0, and, respectively, 10 mM procaine or GH3 effectively reduced the generation of cell membrane lipoperoxides, but GH3 was more effective than procaine especially at the lowest concentration (2.5 mM), when GH3 prevented lipid peroxidation by 21%, versus only 5% for procaine. At 5 and 10 mM, procaine and GH3 showed similar patterns of antioxidant action [29].

Ionizing radiation contributes to ROS generation and DNA damage which is known to be one of the mechanisms responsible for increasing mutagenesis risk, vascular aging, cancer, and neurodegenerative diseases [66–69]. The radioprotective effects of procaine and GH3 (0.25, 0.5, and 1 mM) on the formation of endogenous and X-ray-induced DNA strand breaks in peripheral blood mononuclear cells (PBMCs) isolated from young and elderly individuals were recently investigated. Interestingly, at low concentrations (0.25, 0.5, and 1 mM), GH3 showed the strongest radioprotective effects in PBMCs from young subjects, while procaine reduced the endogenous amount of DNA strand breaks more pronounced in aged individuals. Concentrations of procaine and GH3 of 3 mM and higher (5 and 10 mM) showed a genotoxic effect as measured by DNA strand breaks formation, using the automated fluorescence-detected alkaline DNA unwinding (FADU) assay [29].

4.2. Mitochondria Function. In recent years, it was reported that mitochondria, besides being the main cellular source of ROS, might also be the most relevant target of free radicals, playing a central role in aging [70]. Mitochondria are particularly prone to lipid peroxidation [71, 72], and there is a strong link between mitochondrial metabolism, oxidant species formation, and the biology of aging [73–75]. Mitochondrial ROS also increases with age, and the oxidative stress-dependent decline of cell functions is partially related to the impairment of the mitochondrial respiratory chain [76–78].

As an anesthetic, procaine binds to membrane constituents and modulates a series of ion channels, interacts with membrane phospholipids, and induces concentration-dependent changes in membrane fluidity [42, 79–81]. Moreover, mitochondria, which are considered the powerhouses of the cell, are a potential target for general and local anesthetics [34]. At the cellular and molecular levels, procaine and its metabolites affect several biochemical and cellular processes like membrane conductance [81], oxidative phosphorylation [32], mitochondrial function and structure [33], or fatty acids oxidation [82].

Following an *in vivo* treatment (1 mg procaine/100 g body weight, for 3 days), procaine facilitated oxygen transport towards the mitochondrial matrix by modifying the membrane structure of brain mitochondria in both old and young rats [32]. The mitochondrial ATP-sensitive potassium channel (mitoKATP) is an important component of the mitochondria, whose opening is caused by a calcium signal or by brief episodes of ischemia-reperfusion [34]. de Klaver et al. (2006) demonstrated in human microvascular endothelial cells that tetracaine and procaine had no protective effect against the cell injury induced by inflammation (LPS) and inhibited the activation of mitoKATP channels [83].

Tarba and Crăcium (1990) pointed out, in isolated rat liver mitochondria, a stimulation of the basal state (respiration before ADP addition) in presence of low concentration (1 mM) of procaine. Moreover, procaine had a biphasic effect, exerting a slight stimulation of state-3 respiration (ADP present) at low and moderate concentrations ($\leq$1 mM) and an inhibition at higher concentrations (>1 mM). Besides, electron microscopy confirmed this inhibitory effect, showing an abundance of either swollen or supercondensed mitochondria, with many membrane ruptures. At very low

procaine concentrations (0.01–0.1 mM), the stimulation of the two respiration states is approximately equal and thus the uncoupling effect is absent or negligible [33]. High concentrations (>10 mM) of procaine and GH3 inhibited the uncoupling effect of 2,4-dinitrophenol (2-DNP) on oxidative phosphorylation and stimulated the respiratory activity and induced membrane rigidity thus allowing preferential oxygen diffusion and acceleration of free radical reactions, whereas low concentrations facilitated diffusion of sulfhydryl (SH) containing-compounds, exerting protective effects against lipid peroxidation [84]. Since mitochondrial injury is considered a central event in the early stages of the nephrotoxic effect of the antineoplastic drug cisplatin, Zicca et al. (2002) demonstrated that procaine hydrochloride was able to protect mice and rats through its accumulation in kidneys, followed by coordination with cisplatin (or its hydrolysis metabolites) and formation of a less toxic platinum compound [85]. Previously, Zhang and Lindup (1994) pointed out that procaine (2 mM), DEAE, and PABA protected against the rat kidney cellular damage caused by cisplatin and inhibited by 24, 30, and 22%, respectively, the cisplatin-induced mitochondrial lipid peroxidation, without any changes regarding the mitochondrial protein sulfhydryl groups (protein-SH) [86].

Studies conducted by Onizuka et al. (2010) in rat dorsal root ganglion neurons demonstrated the depolarizing effect of procaine on the mitochondrial membrane by increasing the mitochondrial and intracellular pH, in a dose-dependent manner [87]. Yu et al. (2017) proved that procaine significantly increased neurotoxicity at high concentrations (12, 15, and 20 mM), inducing mitochondrial dysfunction, overproduction of ROS, lipid peroxidation, DNA damage, and apoptosis, in human neuroblastoma cell line SH-SY5Y cells [88].

Recently, using a fluorescent assay with N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), Ungurianu et al. (2020) showed the inhibitory effect of procaine and GH3 on rat liver mitochondria lipid peroxidation providing additional experimental data concerning their antioxidant action in these biological structures. At any of the employed concentrations (0.5, 1.0, 2.0, 5.0, and 10 mM), procaine tended to inhibit lipid peroxidation at higher levels compared to GH3. The inhibitory effect was substantial at the lowest concentration (0.5 mM), 32% for GH3 and 42% for procaine, and increased in a dose-dependent manner. Both procaine and GH3 inhibited at 10 mM more than 80% of the reactive peroxides production in isolated mitochondria fraction [29].

4.3. Cellular Antioxidant Systems—In Vivo Studies. A significant number of studies reported the beneficial effects of *in vivo* treatments with procaine and GH3 in animal models, by modulating the expression of antioxidant enzymes. Thus, chronic treatment with GH3 (20 mg/procaine/kg body weight, three times a week, for nine weeks) modulated lipid peroxidation in homogenates from rat brain tissue as well as $O_2^{\cdot -}$ generation, in correlation with an increase in SOD enzymatic activity [23]. Additionally, procaine and GH3 also induce a significant increase of catalase (CAT) activity in liver and kidney of young, adult, and old rats, and a decrease in the heart of old and adult female rats [89]. Previously, studies using fluorescence and electronic microscopy showed that GH3 treatment decreased lipofuscin (the aging pigment) accumulation in rat brain, testicles, liver, and heart [90, 91].

Procaine as well as procainamide is usually used for the therapy of cardiac arrhythmias. Recently, Qiang et al. (2019) investigated, *in vitro* and *in vivo,* the protective effect of novel 1,3,5-triazine-procaine derivatives against myocardial ischemia-reperfusion injury on the basis of various parameters, such as hemodynamic indices, myocardial enzymes, oxidative stress biomarkers: antioxidant enzymes (SOD, CAT, and glutathione peroxidase [GPx]), glutathione (GSH), hydroxyl radical and superoxide anion scavenging assays, as well as cardiac histopathological examination. Results showed an efficient reduction of ROS, as well as restored to normal levels of GSH and SOD, CAT, and GPx enzymatic activities in the triazine-procaine derivatives-treated group, as compared to myocardial ischemia-reperfusion group. Within this comprehensive experimental model, procaine-1,3,5-triazine derivatives showed significant cardioprotective action via inhibition of nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) [28].

4.4. Lipoprotein Oxidation and Metabolism. Plasma lipoproteins are perfect biological "sensors" of oxidative stress in the arterial wall due to their close interactions with vascular endothelial cells and the high susceptibility of lipids to oxidative alterations [92]. Of particular interest is the impact of oxidative stress on plasma low-density lipoproteins (LDL), as oxidized LDL (oxLDL) are recognized to play a crucial role in promoting atherogenesis by several mechanisms involving their cytotoxicity on monocyte-derived macrophage cells, through reactive species generation and antioxidant failure [93, 94]. Therefore, LDL are the most targeted by oxidative stress associated with metabolic imbalances such as hyperlipidemia, hyperglycemia, or insulin resistance. Increased LDL oxidation and development of a low-grade proinflammatory environment have been proposed to contribute to age-dependent endothelial dysfunction [94].

Gradinaru et al. (2009) studied *in vitro* the effect of procaine and GH3 on LDL oxidation, by incubating native LDL isolated from human plasma with 0.1, 0.5, and 1 mM procaine/GH3. The kinetic analysis of conjugated dienes formation during the Cu^{2+}-induced oxidation of LDL revealed that 1 mM procaine significantly inhibited LDL oxidation at 20 and 60 minutes after its inducement, whereas 1 mM GH3 exerted a long-lasting antioxidant effect, with a significant inhibition even after 180 minutes [25].

Ungurianu et al. (2020), using a sensitive-fluorescent assay with Amplex Red, also pointed out, in human serum lipoprotein concentrates, a dose-dependent inhibitory action of procaine and GH3 on lipid peroxidation, significant at all tested concentrations (0.5, 1.0, 2.0, 5.0, and 10 mM). GH3 showed a significantly higher lipid peroxidation inhibition compared to procaine. Additionally, the effect of GH3 and procaine treatment was examined on cell-mediated LDL oxidation induced in human-derived U937 cultured macrophages. Cellular oxidative stress was evaluated using the thiobarbituric acid reactive substances (TBARS) released in

the incubation medium, as oxidative stress biomarker for the global measurement of lipid peroxidation end-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). At all the tested concentrations (0.5, 1.0, and 2.0 mM), GH3 significantly decreased TBARS (%), whereas the effect of procaine was lower, reaching only half of GH3 effect at 2 mM [29].

Overall, procaine's significant antioxidant action demonstrated by *in vitro* and *in vivo* studies may contribute to its geroprotective effect, but the question is which are the molecular mechanisms for procaine's antioxidant effect? It could act as an inhibitor of the chain reactions generating the lipid peroxides, or/and as "scavenger" of the ROS. While procaine was more effective in protecting the cellular and mitochondrial membranes, GH3 was more efficient against serum lipoperoxidation. These outcomes could be explained through the different lipid or lipoprotein microenvironments present in these biological systems and/or through the different intrinsic antioxidant capacities or ROS scavenging actions of procaine and GH3, in counteracting or preventing lipid peroxidation [29].

The main limitation of these studies is that they present overall effects and did not investigate the specific molecular and cellular mechanisms involved, such as the modulation of signaling pathways which can result in enhancing cell antioxidant response. Therefore, further investigations are needed to establish the exact mechanisms of action of procaine and GH3.

Several experimental studies have highlighted the role of procaine as a modulator of lipid metabolism [95, 96]. The lipid-lowering action of procaine was explained via a "statin-like" action exerted through the regulation of 3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol biosynthesis [35]. The effect of procaine on steroidogenesis was reported in human H295R adrenal cells and in procaine-treated rats. This inhibitory activity was not observed in Leydig cells, suggesting that the effect could be specific to adrenocortical cells. Procaine did not affect either cyclic AMP- (cAMP-) dependent protein kinase activity, key proteins involved in mitochondrial cholesterol transport, side-chain cleavage enzymes, or enzymatic activities associated with the final stage of cholesterol biosynthesis. However, procaine reduced HMG-CoA reductase activity and specific mRNA expression dose-dependently. The modulatory effect of procaine on HMG-CoA reductase mRNA was also observed in the Hepa 1-6 mouse hepatoma cells stimulated by dibutyryl-cyclicAMP (dbcAMP) [35].

Procaine was also tested *in vitro* for its action as an inhibitor of the two enzymes involved in cholesterol esterification: acyl-CoA cholesterol acyltransferase (ACAT) and lecithin-cholesterol acyltransferase (LCAT) [97, 98]. Bell and Hubert (1981) used a microsomal fraction isolated from rabbit aorta, in which they monitored the incorporation of [^{14}C]-oleyl-SCoA in the form of [^{14}C]-cholesterol esters. The ACAT activity was inhibited depending on the concentration of anesthetic (0.25-0.50 mM procaine) in the reaction medium [97]. Another experiment was performed *in vitro* in human, rat, and dog plasma samples incubated with 1-5 mM procaine, which significantly inhibited LCAT activity [98]. Some longitudinal and clinical studies carried out in elderly subjects with systemic atherosclerosis evidenced the lipid-lowering and antioxidant actions of GH3 treatment [24, 30].

These results lead to a complex pharmacological profile of procaine, which includes the modulation of cholesterol metabolism at all levels, from genetic control of sterol biosynthesis to its esterification in plasma and tissues, with potential clinical applications in the treatment of hypercholesterolemia. Moreover, elevated glucocorticoid levels are associated with many pathologies, including age-related depression, hypertension, Alzheimer's disease (AD), or acquired immunodeficiency syndrome, cortisol biosynthesis reducing agents being a possible useful complementary therapy for all these conditions.

All these data may suggest that a combination of GH3 with lipid-lowering drugs could diminish the doses and the adverse effects of the classical treatment of hyperlipidemia.

5. Cerebral Age-Related Pathology

Similar to the structurally related cocaine, the effects of procaine go beyond its anesthetic actions, with some concerning the central nervous system (CNS). Several beneficial effects of procaine and GH3 were reported, such as ameliorating depression and cognitive abilities (conditioned behavior, memory) and increasing cerebral resistance to different aggressive actions (acute intoxication, hypoxia, and electric shock) [36, 37, 99–105].

Monoamine oxidases (MAOs), a class of enzymes involved in the metabolism of catecholamines and other biogenic amines, are increasingly recognized as major contributors to the generation of mitochondrial ROS. The best-known and characterized MAOs are the endothelial and neuronal ones. However, the inducible isoforms which can be expressed in various tissues and organs have lately gained notoriety and stimulated interest in the extracerebral roles of these enzymes. For an overview of the complex roles of MAOs in age-associated diseases, the reader is referred to a recent review by Santin et al. (2021) [106]. It is well known that the expression of human MAOs and their abilities to produce ROS increase with age (4-fold MAO-B in neuronal tissue and 6-fold MAO-A in the heart) and are involved in the etiology of age-associated chronic pathologies: depressive disorders, Parkinsonism, cardiac diseases, and diabetes [107, 108].

In 1940, Philpot evidenced for the first time, using rat liver homogenate, procaine's inhibitory effect on the tyramine and adrenaline oxidation, at high concentrations (33 mM procaine) in the reaction mixture [109]. Further studies pointed out MAO inhibitory actions for both procaine and GH3 in brain, liver, and heart tissues from mice and rats [110, 111]. The MAO B inhibitory action of procaine was reported following pharmacodynamics studies, and GH3 was included in the category of reversible and competitive inhibitors [112, 113]. The inhibition of MAO activity by local anesthetics depends on both electrostatic and hydrophobic interactions between these drugs and enzyme-associated phospholipids or the hydrophobic regions of proteins [114]. MAO inhibitory effect was associated with the inhibition of

lipid peroxidation in rat brain homogenates and mitochondrial fraction, pointing out that MAO activity could be inhibited through a limitation of free radical reactions [115].

Using rat pheochromocytoma PC12 cells, Lecanu et al. (2005) observed that procaine is a ligand of the sigma 1 receptor, a protein whose ligands have been shown to protect mitochondrial function and to exert antidepressant properties. Procaine also displayed strong neuroprotective properties against the amyloid peptide $A\beta_{1\text{-}42}$ and preserved $A\beta_{1\text{-}42}$-induced ATP depletion. Procaine inhibited the neurotoxic effect of glutamate on PC12 cells, suggesting that the reduction of glutamate-induced neurotoxicity may be the mechanism by which procaine exert its "anti-amyloid" effect [116]. Li et al. (2016) studied the effect of procaine treatment in a rat model of neuropathic pain. Procaine inhibited Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) expression, both mRNA and protein levels, indicating its cellular mechanism in attenuating neuropathic pain [117]. Recently, Wu et al. (2020) synthesized a series of procaine-imidazole derivatives with potent and selective MAO-B inhibitory activity, as well as *in vivo* anti-Parkinson effects using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease. These procaine-based compounds determined a significant improvement of motor function in mice as revealed by motor behavioral assessment using the footprint and horizontal wire test and also improved the level of antioxidant enzymes in the striatum of animal brains [37].

Older studies regarding the effects of GH3 treatment on the function of CNS reported an improvement of cognitive function, better work capacity, increased resistance to stress and effort, decrease of depression symptoms, and an overall higher capacity of adapting to external stimuli. These data underlined that geriatric procaine-based products have minimum/short-lived side effects compared to classic CNS-targeted medication, tolerated with difficulty by elderly with polypathology [99, 100, 110, 118, 119]. For decades, procaine infusions have been applied in patients with psycho-vegetative disturbances, mostly during neural therapy—a common complementary treatment approach using injections with short-acting local anesthetics to treat pain and chronic diseases. However, little is known about the underlying mechanisms and the domains of treatment response [102, 103].

Hahn-Godeffroy et al. (2019) studied, in 56 case-control patients, the effect of intravenous procaine (1-3 ampoules of 5 ml of procaine 2% in 250 ml sodium chloride per treatment setting) on the somatic and psycho-vegetative state of health. After 4 or 6 months, 75% of patients showed an improvement in the 9 positive items, e.g., "hedonia," "joyness," or "improved sleep." 62.5% of patients reported a substantial attenuation in the 12 negative items, e.g., "stress reactions," "loss of energy," or "anxiety." All these changes were significant after 2, 4, and 6 months compared to the values at baseline, suggesting a long-lasting improvement of somatic and psycho-vegetative symptoms under the infusion of procaine alone, which modulates the activities of specific brain areas such as the limbic system [102].

Haller et al. (2018) performed, in 22 patients with multiple diagnoses, a qualitative analysis of self-reported outcomes following neural therapy injections with procaine. Patients experienced an emotional release and physical symptoms relief, consisting of improved mood, increased pain acceptance, and empowerment. Adverse events of neural therapy included pain at the injection site, vegetative complaints, and emotional turmoil that lasted for minutes or hours, with a maximum of two days [103]. Oettmeier et al. (2019) reported the clinical use of a highly-dosed infusion of procaine hydrochloride with sodium bicarbonate as an additive, for the treatment of different acute diseases, chronic pain, and inflammations [31].

Xu et al. (2016) explored the effects of daily use of GH3 tablets, for three months, on relieving mental symptoms and improving health-related quality of life among Chinese older adults. The randomized, placebo-controlled, double-blinded study comprised 100 eligible participants, men and women between 50 and 89 years of age. GH3 treatment showed positive outcomes in supporting mental health and improving general health and well-being, while promoting the recovery of cognitive function among older adults. Average levels of low mood and anxiety concerns (evaluated with Self-Rating Depression and Anxiety Scales) were both reduced, and the prevalence rate of clinical anxiety was decreased [104].

6. Negative Results Reported in Experimental and Clinical Studies

The use of procaine for nonlocal-anesthetic purposes is highly controversial, especially when employed for its alleged "anti-aging effects" [15]. Different pharmaceutical preparations, including GH3, were widely promoted and commercially available "over the counter" in any country, especially via online marketing. Also, there are reports of studies which comparatively examined the effects of procaine (and GH3) and other anesthetics in different age-related maladies reported a lack of efficacy, as well the presence of toxicity or even severe side-effects [13, 14, 120–123].

Several experimental and clinical studies described significant neurologic alteration following procaine and GH3 treatment. In a review commissioned by the National Institute on Aging of the National Institutes of Health (USA), Ostfeld et al. (1977) evaluated scientific literature on the systemic use of procaine in the treatment of the aging process and the common chronic diseases, including data from 285 articles and books, describing the treatment in more than 100,000 patients. Except for a possible antidepressant effect, they found no convincing evidence that procaine or GH3 has any value in the treatment of aging-associated diseases in older patients [13] In a systematic review, Szatmari and Bereczki (2008) assessed independently the efficacy and adverse effects of procaine and GH3 treatment regarding cognitive function improvement in subjects with dementia from randomized, double-blind trials carried out before the 1990s. Pooling data from two studies showed a detrimental effect of procaine in terms of causing side effects. In patients with dementia, a single small study also suggested a negative effect, while two trials including healthy elderly individuals suggested a positive effect of procaine use on cognitive

function. Authors concluded that the evidence for detrimental effects of procaine and its preparations is stronger than the data reporting its benefits in preventing and/or treating dementia or cognitive impairment [14].

Zaric and Pace (2009) searched the Cochrane Central Register of Controlled Trials for frequency of transient neurologic symptoms (TNS—painful condition that occurs in the immediate postoperative period) and neurologic complications after spinal anesthesia with lidocaine compared to other local anesthetics, including procaine. The risk of developing TNS after spinal anesthesia with lidocaine was significantly higher than when bupivacaine, prilocaine, or procaine were used [123]. Ghafari et al. (2012) evaluated, in a prospective, randomized, double-blind trial, comprising 110 patients (aged between 20 and 70 years), and the effect of lidocaine versus procaine on cognitive impairment manifested after coronary artery surgery. In the procaine group, the neurocognitive total score decreased significantly compared to the preoperative score and compared to the lidocaine group [122].

Takenami et al. (2012) aimed to compare the neurotoxicity of intrathecal procaine, bupivacaine, levobupivacaine, and ropivacaine in a rat spinal model. Although the four local anesthetics seemed to cause identical neurotoxic lesions commencing in the posterior root and extending to the posterior column by axonal degeneration, bupivacaine appeared to be the most neurotoxic of the four drugs, and the neurotoxicity at higher doses increased by administered volume with procaine > levobupivacaine > ropivacaine [121]. Yilbas et al. (2018) studied the effect of intra-articular procaine injection on knee articular cartilage and the synovium of Sprague-Dawley rats. Results showed no significant differences in inflammation (using a histological evaluation) between procaine and saline (control) groups at any duration of treatment (after 1, 2, 7, 14, and 21 days). No significant difference was detected in the percentage of apoptotic chondrocytes between groups at any of the time intervals [120].

7. DNA Methylation and Tumorigenesis

DNA methylation is an epigenetic modification involved in gene expression regulation. Age-associated alterations in DNA methylation are commonly grouped in the phenomenon known as "epigenetic drift," which is characterized by gradual extensive demethylation of genome and hypermethylation of a number of promoter-associated $5'$-cytosine-phosphate- guanine-$3'$ (CpG) islands. For an overview on the reconfiguration of DNA methylation in aging the reader is referred to a recent review by Zampieri et al. (2015) [124]. Possible consequences are mutations and dysregulation of gene expression, which can either lead to cell death or cellular senescence or to malignant transformation of the cells, ultimately resulting in cancer [125]. DNA methyltransferases (DNMTs) are a family of enzymes that methylate DNA at the C5 position of cytosine followed by a guanine residue (CpG dinucleotide). Reexpression of methylation silenced tumor suppressor genes by inhibiting the DNMTs (DNMT1, DNMT3A, and DNMT3B) has emerged as an effective strategy against cancer [126]. A large number of preclinical studies have shown that local anesthetics have a direct inhibitory effect on tumor activities, including cell survival, proliferation, migration, and invasiveness [41, 127, 128]. Recently, Moreira-Silva et al. (2020) cited procaine among the "repurposed drugs" which have demonstrated promising results as epigenetic inhibitor in *in vitro* tumorigenesis [129].

DNA hypermethylation and the consequent silencing of tumor suppressor genes are considered as a molecular hallmark of many kinds of cancers. Villar-Garea et al. (2003) demonstrated for the first time the role of procaine as a DNA demethylating agent in breast cancer cells, evidencing a 40% reduction in 5-methylcytosine (5mC) DNA content. Procaine had also the capacity to demethylate densely hypermethylated CpG islands, such as those located in the promoter region of the retinoic acid receptor (RAR) beta2 gene, restoring gene expression of epigenetically silenced genes. Finally, procaine also had growth-inhibitory effects in these cancer cells, causing mitotic arrest [38]. Gao et al. (2009) provided the first evidence that procaine is able to reactivate, in lung cancer cells, the Writ inhibitory factor-1 (WIF-1), which was silenced due to promoter hypermethylation [130].

Using hepatoma cells and nude mice bearing xenograft, Tada et al. (2007) revealed that procaine displayed both growth-inhibitory and demethylating effects on human hepatoma cells, both *in vitro* and *in vivo*. All the genes transcriptionally suppressed by DNA hypermethylation were demethylated and reactivated following procaine treatment. Morphological observations showed a significant reduction in tumor volume *in vivo* [131]. Castellano et al. (2008), who synthesized several analogues of procaine and tested their inhibiting activity against DNMT1, discovered a derivative able to induce a recognizable demethylation of chromosomal satellite repeats in HL60 human myeloid leukemia cells [132].

Another mechanism underlining procaine's anticancer activities is through direct interaction with DNA. In a multispectroscopic and molecular modelling study, Ali et al. (2018) used molecular docking on five different B-DNA structures (extracted from the Protein Data Bank) and showed that procaine binds to the adenine-thymine (AT) rich region of all five calf thymus B-DNA structures. Simultaneously, they found that procaine acts as an electron donor to DNA bases when testing the anticancer activity of procaine alone and in combination with doxorubicin in MCF-7 breast cancer cells [133].

Procaine also demonstrated nonepigenetic effects, such as the inhibition of cell proliferation and migration, and also enhancement of apoptosis in gastric cancer cells, osteosarcoma cells, colon cancer cells, mouse models of lung cancer, human leukemia cells, and human bladder cancer cells [134–139]. Borutinskaite et al. (2016) examined the effects of procaine as DNMT inhibitor on growth inhibition, apoptosis, and differentiation of human leukemia cells and showed an increase in the expression of molecules associated with differentiation, such as integrin CD11b, E-cadherin, granulocyte colony-stimulating factor (G-CSF), and apoptosis-peroxisome proliferator-activated receptor (PPAR)

Table 1: Molecular and cellular effects of procaine reported within *in vitro* and *in vivo* studies, which support its antioxidant action.

Preclinic model	Target	Concentrations/doses	Relevant finding	Reference
In vitro superoxide ($O_2^{\cdot-}$) generation	Nonenzymatic system: [NADH-PMS-NBT]	0.2 to 2.0 mM procaine/GH3	$O_2^{\cdot-}\downarrow$ procaine; $\downarrow\downarrow$ GH3	Rusu and Lupeanu (1989) [22]
In vivo treatment	Rat tissue homogenates Rat tissue samples histopathological analysis	20 mg procaine/kg body weight, 3 times/week × 9 weeks	$O_2^{\cdot-}\downarrow$ lipid peroxidation $\rightarrow$ (liver, brain, kidney) $\downarrow$SOD activity (brain) $\uparrow$CAT activity (liver, kidney) Lipofuscin $\downarrow$ (brain, testicles, liver, heart)	Rusu et al. (1992) [23] Lupeanu (1999) [89] Radaceanu et al. (1991) [90]
In vitro sepsis	Neutrophils + LPS, triggered with fMLP	4.0 mM procaine	$\downarrow$ 50% LPS priming $\downarrow$ LPS-induced up-regulation, cytochrome b558	Jinnouchi et al. (2005) [64]
In vitro superoxide ($O_2^{\cdot-}$) generation	Enzymatic system: [xanthine – XO – INT]	1.0 to 10.0 mM procaine/GH3	$O_2^{\cdot-}\downarrow$ procaine; $\downarrow\downarrow$ GH3	Gradinaru et al. (2009) [25]
In vitro total antioxidant capacity	ABTS cation + different local anesthetics	10 mM	Scavenging (%): tetracaine > procaine > lignocaine > benzocaine (99; 38; 21; 20%)	Librowski and Moniczewski (2010) [27]
In vitro ROS exposure	Isolated rabbit aortic rings	10^{-5}M to 3×10^{-3} M procaine/lidocaine	Endothelium-dependent vasorelaxation $\uparrow\downarrow$	Lee et al. (2010) [26]
In vitro sepsis	Bovine aortic endothelial cells + IL-1β/LPS	10 mM procaine	$\downarrow$ NO production	Takaishi et al. (2013) [65]
In vivo and *in vitro* myocardial ischemia-reperfusion injury	Rat tissue samples	1,3,5-triazine-procaine derivatives	Cardioprotective $\downarrow\downarrow$ ROS $\uparrow$ GSH, SOD, CAT, and GPx $\downarrow$ LOX-1 Antiapoptotic $\downarrow$ Bax; $\uparrow$ Bcl-2 Anti-inflammatory $\downarrow$NF-κB,	Qiang et al. (2019) [28]
	RAW264.7 macrophages transfected with NF − κB + LPS	5 and 10 mg/mL, in K-H buffer solution x 45 min 100 mM		
In vitro ROS exposure	Jurkat cells + CuOOH	2.5 to 10 mM procaine/GH3	Membrane lipoperoxides $\downarrow$ procaine; $\downarrow\downarrow$ GH3	Ungurianu et al. (2020) [29]
In vitro X-ray-DNA damage in human lymphocytes	PBMCs, young, and elderly subjects	0.25 to 10 mM procaine/GH3	Radioprotective (0.25 to 1 mM) GH3 $\downarrow$ DNA damage, young Procaine $\downarrow$ endogenous DNA strand breaks, aged Genotoxic (3 to 10 mM)	Ungurianu et al. (2020) [29]

ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; CAT: catalase; CuOOH: cumene hydroperoxide; fMLP: N-formyl-methionyl-leucyl phenylalanine chemotactic peptide; GH3: Gerovital H3; GPx: glutathione peroxidase; GSH: reduced glutathione; IL-1β: interleukin-1 beta; INT: 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; LOX-1: lectin-like oxidized low-density lipoprotein receptor-1; LPS: lipopolysaccharide; NADH: reduced nicotinamide adenine dinucleotide; NBT: nitroblue tetrazolium; NF-κB: nuclear factor kappa light chain enhancer of activated B cells; NO: nitric oxide; PBMCs: peripheral blood mononuclear cells; PMS: phenazine methosulfate; ROS: reactive oxygen species; SOD: Cu/Zn-superoxide dismutase; XO: xanthine oxidase.

gamma. Moreover, procaine enhanced certain gene transcription activation via chromatin remodeling—the changes in histone H3K4 (Me)3 and H3K9Ac/S10P modifications were detected [138]. Sun et al. (2012) proved that procaine might be used as a potential agent for bladder cancer treatment as it inhibited the proliferation of T24 and 5637 human bladder cancer cells by inducing their apoptosis. The mechanism studies reveal that procaine could induce demethylation of apoptotic peptidase activating factor 1 (APAF1) gene in T24 or 5637 cells, subsequently activating caspase-3/9. It was also shown that the serum soluble fas ligand (sFasL) was activated, and the expression of matrix metallopeptidase 9 (MMP-9) was downregulated [139]. The common mechanism by which procaine inhibited cancer cell proliferation and migration was the inactivation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK)/focal nuclear adhesion kinase (FAK) and protein kinase B (AKT)/extracellular signal-regulated kinase (ERK) pathways [134, 135]. In a recent study, Fan et al. (2021) identified a novel mechanism through which procaine can impair the survival and self-renewal of the malignant glioblastoma stem cells, suggesting that local anesthetics may weaken zinc finger Asp-His-His-Cys-type palmitoyltransferase 15 (ZDHHC15) transcripts and decrease glycoprotein 130 (GP130) palmitoylation levels

Table 2: Molecular and cellular effects of procaine on mitochondria function, reported within *in vitro* studies.

Preclinic model	Target	Concentrations/doses	Relevant finding	Reference
In vitro	Rat liver mitochondria	0.5 to 10 mM procaine/GH3	Procaine (1 mM) ↑ basal state, respiration before ADP addition Procaine (>10 mM) ↓ 2,4-DNP, oxidative phosphorylation Lipid peroxidation ↓ ↓ procaine ↓ GH3	Tarba and Cracium, 1990 [33] Rusu (1990) [84] Borsa et al. (2002) [115] Ungurianu et al. (2020) [29]
In vitro cisplatin-induced nephrotoxicity	Rat renal cortical slices	2 mM procaine	↓ mitochondrial injury ↓ cellular damage ↓ lipid peroxidation	Zhang and Lindup (1994) [86]
In vitro sepsis	Human vascular endothelial cells + LPS	0.01 to 1.0 mM procaine	→ cell injury ↓ mitoKATP activation	de Klaver et al. (2006) [83]
In vitro	Rat dorsal root ganglion neurons	1, 5, and 10 mM procaine	↑ depolarization of the mitochondrial membrane potential ($\Delta\Psi$m) ↑ [pH]m	Onizuka et al. (2010) [87]
In vitro neuroblastoma	Human cell line SH-SY5Y	12, 15, and 20 mM procaine	↑ neurotoxicity, ↑ mitochondrial dysfunction, ↑ ROS ↑ lipid peroxidation ↑DNA damage and apoptosis	Yu et al. (2017) [88]

2,4-DNP: 2,4-dinitrophenol; GH3: Gerovital H3; mitoKATP: mitochondrial ATP-sensitive potassium channel; ROS: reactive oxygen species.

and membrane localization, thus, inhibiting the activation of interleukin-1 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling [41].

As to procaine's effects in normal cells, Schumann et al. (2020) characterized the action of procaine and S-adenosyl-L-homocysteine (SAH) as demethylating agents, on the expression of genes related to the epigenetic machinery, including the DNMTs and on DNA methylation levels in bovine skin fibroblasts. Global DNA methylation levels were significantly lower in cells that were cultivated in medium containing both compounds versus control cells, and gene expression of DNMT1, DNMT3A, and DNMT3B decreased significantly in cells cultivated with SAH + procaine (1 mM). Moreover, a significant decrease in DNMT3B transcript levels was found in cells cultivated with procaine. Higher levels of the ten-eleven translocation enzyme-3 (TET3) dioxygenase, involved in the epigenetic machinery, were also found in cells cultivated with procaine and SAH + procaine, compared with the control [40].

Using a mouse behavioral sensitization model in which animals were subjected to an acute treatment with procaine for seven days, Anier et al. (2018) found that procaine caused a decrease on the DNMT3A mRNA levels in peripheral blood cells (PBCs), suggesting that the inhibition of voltage-gated sodium channels may be the mechanism that alters DNMT expression in PBCs [39].

8. Procaine Effects on Lifespan

The studies researching procaine's influence on lifespan are scarce. An experimental study conducted by Aslan et al. (1965) on 1840 rats pointed out 18-21% longer lifespan in treated animals than that of controls injected with saline solution [140]. Another investigation on lifespan was conducted on 3680 animals from 5 successive generations. The outcome supported that GH3 administered since early ages induced a lifespan extension both in the treated animals, as well as in the first generation of not-treated offspring [141]. In *Drosophila melanogaster* grown on nutritive medium enriched with GH3 was also found out a 22.7% lifespan extension, compared with controls [142]. Unfortunately, there are no recent studies regarding the effects of procaine and/or GH3 on lifespan.

9. Conclusions and Perspectives

The analysis of older and more recent (between 1990 and 2020) literature data reveals the diversity of procaine's effects at cellular and molecular levels, in preclinical studies and clinical settings.

Summarizing its cellular actions, procaine is able to bind to membrane constituents and interact with a series of ion channels exerting its anesthetic action [42, 143] and also has a significant influence on oxidative stress response, on the modulation of critical metabolic pathways, as well as on epigenetic regulation.

The antioxidant action of procaine is supported by *in vitro* and *in vivo* experimental studies regarding the inhibition of ROS generation and lipid peroxidation, in enzymatic [25] and nonenzymatic systems [23, 27], associated with a modulating effect on antioxidant enzymes [23, 28, 89]. Several studies confirm its involvement in mitigating cellular and systemic oxidative stress, acting on the main targets of aging and age-related diseases: cell membranes [22, 24], lipoproteins [25, 29], mitochondria [29, 37, 86], and DNA [29]. Procaine reaffirmed its antioxidant and cytoprotective actions in experimental models of myocardial ischemia/-reperfusion injury [28, 83], endothelial-dependent vasorelaxation [26], inflammation [65], sepsis [64], ionizing irradiation [29], and intoxication [85, 86] (Table 1).

Table 3: Molecular and cellular effects of procaine on lipoprotein oxidation and metabolism, reported within *in vitro* and *in vivo* studies, which support its antiatherogenic action.

Preclinic model	Target	Concentrations/doses	Relevant finding	Reference
In vitro and *in vivo* treatment	Human H295R adrenal cells, Hepa 1-6 mouse hepatoma cells	0.1, 1, 10, and 100 μM procaine, for 48 h	↓ steroidogenesis ↓ HMG-CoA reductase activity	Xu et al. (2003) [35]
	Rats	25–100 mg procaine/kg body weight, 8 days	↓ mRNA expression ↓ serum corticosterone	
In vitro treatment	Human, rat, dog plasma	1–5 mM procaine	↓ plasma LCAT	Bell and Hubert (1980) [97]
	Rabbit aorta microsomes	0.25–0.50 mM procaine	↓ ACAT	Bell (1981) [98]
In vitro LDL oxidation	Human plasma LDL + Cu^{2+}	0.1–1.0 mM procaine/GH3	Conjugated dienes ↓ procaine; ↓↓ GH3	Gradinaru et al. (2009) [25]
In vitro LDL oxidation	U937 macrophages + human plasma LDL + Cu^{2+}	0.5–2.0 mM	TBARS ↓ procaine; ↓↓ GH3	Ungurianu et al. (2020) [29]
In vitro lipoprotein oxidation	Human serum lipoprotein concentrates	0.5 to 10 mM procaine/GH3	Lipid peroxidation: ↓ procaine; ↓↓ GH3	Ungurianu et al. (2020) [29]

ACAT: acyl-CoA cholesterol acyltransferase; GH3: Gerovital H3; HMG-CoA: 3-methylglutaryl-coenzyme A; LCAT: lecithin-cholesterol acyltransferase; LDL: low-density lipoproteins; TBARS: thiobarbituric acid reactive substances.

Table 4: Molecular and cellular effects of procaine reported within *in vitro* and *in vivo* studies, which support its neuroprotective actions.

Preclinic model	Target	Concentrations/doses	Relevant finding	Reference
In vivo treatment	Rat brain and liver mitochondria	60 mg procaine/kg body weight, 5 times/week × 4 weeks	MAO activity: ↓ procaine; ↓↓ GH3	Borsa et al. (2002) [115]
In vitro β-amyloid-induced neurotoxicity	Rat pheochromocytoma PC12 cells	1, 10, and 100 μM procaine	↓ amyloid peptide Aβ1-42 ↓ amyloid -induced ATP depletion ↓ glutamate neurotoxic effect	Lecanu et al. (2005) [116]
In vivo neuropathic pain	Rat tissue samples of L4-L6 spinal dorsal horn Behavior tests	2% procaine intrathecal injection in DMSO (10 μL/kg)	↓ JAK2 ↓STAT3 (mRNA+protein) ↓ pain behavior	Li et al. (2016) [117]
In vivo MPTP-induced Parkinson's disease	Rat liver mitochondria Mouse brain homogenate Behavior tests	Procaine-imidazole derivatives 25, 50, and 100 mg/kg body weight, 3 days	↓ MAO-B ↑ striatum antioxidant enzymes ↑ motor function	Wu et al. (2020) [37]

GH3: Gerovital H3; DMSO: dimethyl sulfoxide; JAK2: Janus kinase 2; MAO: monoamine oxidase; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; STAT3: signal transducer and activator of transcription 3.

Like many pharmacologic active molecules, procaine exhibits a multimodal dose response. In low concentrations (≤1 mM), protective effects were reported regarding mitochondrial function, lipid peroxidation, and DNA damage, whereas high concentrations (>10 mM) induced membrane rigidity, acceleration of free radical reactions, genotoxicity, neurotoxicity, mitochondrial dysfunction, and apoptosis [29, 33, 87, 88] (Table 2).

Procaine might exert its actions regarding the atherogenesis process by modulating lipoprotein metabolism, as an inhibitor of the key enzymes involved in cholesterol biosynthesis and esterification: HMG-CoA reductase, ACAT, and LCAT [35, 97, 98], and via its antioxidant mechanisms, reducing the oxidative stress exerted on the LDL [25, 29] (Table 3).

Numerous beneficial actions were reported for procaine concerning the CNS, beyond its anesthetic effect. Experimental studies highlighted neuroprotective, antidepressant, and "anti-amyloid" actions [116], along inhibition of JAK2 and STAT3 expression in neuropathic pain models [117]. The inhibitory effect on MAO-B of procaine-derivatives, as well as the *in vivo* anti-Parkinson effect, was associated with lower levels of mitochondrial lipid peroxidation and improved levels of antioxidant enzymes in the striatum [37] (Table 4).

Various recent clinical studies pointed out the functional improvement of somatic and psycho-vegetative symptoms during neural therapy with procaine [102, 103]. GH3 treatment shows positive effects in supporting mental health and improving general health and well-being, while promoting the recovery of cognitive function among older adults [104].

Procaine could be considered as a reference substance for DNA-demethylation and tumor-suppressive effects, although these interventions may only be detectable in specific types of cancer due to differential methylation profiles [144].

Table 5: Molecular and cellular effects of procaine as DNA demethylation and tumor-suppressive agent, reported within *in vitro* studies.

Preclinic model	Target	Concentrations/doses	Relevant finding	Reference
Breast cancer	Human MCF-7 cell line	0.005–0.5 mM procaine, 72 h	↓ 40% DNA 5mC ↓*RARβ2* gene CpG islands ↑ mitotic arrest	Villar-Garea et al. (2003) [38]
Lung and colon cancer	Human H460, A549, and HCT116 cells	2 mM procaine	↑ silenced WIF-1	Gao et al. (2009) [130]
Hepatocellular carcinoma	Human hepatoma cells and nude mice bearing xenograft	1 mM procaine, 5 days	↑ mitotic arrest ↓ CpG islands ↓ tumor volume	Tada et al. (2007) [131]
Leukemia	Human myeloid HL60 cells	Procaine analogues 0.5 mM, 72 h	↓ DNMT1 ↓ CpG islands	Castellano et al. (2008) [132]
Bladder cancer	Human T24 and 5637 cells	5 – 10 μM procaine	↓ proliferation ↑ apoptosis ↔ APAF1 gene demethylation ↑ caspase-3/9; ↑ sFasL; ↓ MMP-9	Sun et al. (2012) [139]
Breast cancer	Human BT-20 (ER-negative) and MCF-7 (ER-positive) cell lines	Procaine and lidocaine, 0.01; 0.1, and 1 mM, 72 and 96 h	↑ apoptosis ↓ DNA 5mC	Lirk et al. (2012) [144]
Lung cancer	Mouse lung cancer with A549 and NCI-H1975 xenograft	50 mg procaine/kg body weight × 3 weeks	↓ tumor proliferation	Ma et al. (2016) [137]
	Human nonsmall cell lung cancer A549 and NCI-H1975 cell lines	100 nM procaine	↓ cell proliferation ↓ EGFR mRNA	
Leukemia	Human NB4 cells	3–5 μM procaine	↑ CD11b, E-cadherin, G-CSF ↑ PPAR gamma ↔ chromatin remodeling—histone H3K4(Me)3 and H3K9Ac/S10P	Borutinskaite et al. (2016) [138]
Osteosarcoma	Human MG63 cells	2 μM procaine	↓ proliferation and migration ↑ apoptosis miR-133b upregulation AKT/ERK inactivation	Ying et al. 2017 [135]
Colon cancer	Human HCT11 cells	0.5, 1, 1.5, and 2 μM procaine 3 μM procaine + carboplatin 5 μM procaine	↓ proliferation and migration ↑ apoptosis ↑ RhoA expression ↑ DNA fragmentation ↓ DNA 5mC	Li et al. (2018) [136] Sabit et al. (2016) [145]
Mouse behavioral sensitization model	Peripheral blood cells	1–10 μM procaine	↓ DNMT3A mRNA	Anier et al. (2018) [39]
Gastric cancer	Human SGC-7901 and GES-1 cell lines	1–5 μM procaine	↓ DNMT1/DNMT3A activity ↓ proliferation ↑ apoptosis CDKN2A and RAR upregulation	Li et al. (2018) [136]
Molecular docking on B-DNA structures	Calf thymus	5–35 μM procaine	↔ binding to AT rich regions → electron donor to DNA bases	Ali et al. (2018) [133]
Breast cancer	Human MCF-7 cell line	5 μM procaine	→ anticancer activity	
Normal cells	Bovine skin fibroblasts	Procaine + SAH (1 mM)	↓ DNMT1, DNMT3A, DNMT3B ↓ DNMT3B (procaine) ↑ TET3 dioxygenase	Schumann et al. (2020) [40]

Table 5: Continued.

Preclinic model	Target	Concentrations/doses	Relevant finding	Reference
Brain cancer	Human glioblastoma stem cells	5, 10, and 20 μM procaine	↓ survival and self-renewal ↓ ZDHHC15 transcripts ↓ GP130 palmitoylation ↓ activation of IL-6/STAT3	Fan et al. (2021) [41]

5mC: 5-methylcytosine; AKT: protein kinase B; APAF1: apoptotic peptidase activating factor 1; AT: adenine-thymine; CDKN2A: cyclin dependent kinase inhibitor 2A; CpG: 5′-cytosine-phosphate-guanine-3′; DNMT: DNA methyltransferase; EGFR: epidermal growth factor receptor; ERK: extracellular signal-regulated kinase; ER: oestrogen receptor; G-CSF: granulocyte colony-stimulating factor; GP130: glycoprotein 130; H3K4 (Me)3: tri-methylation at the 4th lysine residue of the histone H3 protein; H3K9Ac/S10P: phospho-acetylated histone H3; miR: microRNA; MMP-9: matrix metallopeptidase 9; PPAR: peroxisome proliferator-activated receptor; RAR: retinoic acid receptor; RhoA: Ras homolog family member A; SAH: S-adenosyl-L-homocysteine; sFasL: serum soluble fas ligand; STAT3: signal transducer and activator of transcription 3; TET3: translocation enzyme-3; WIF-1: Writ inhibitory factor-1; ZDHHC15: zinc finger Asp-His-His-Cys-type palmitoyltransferase 15.

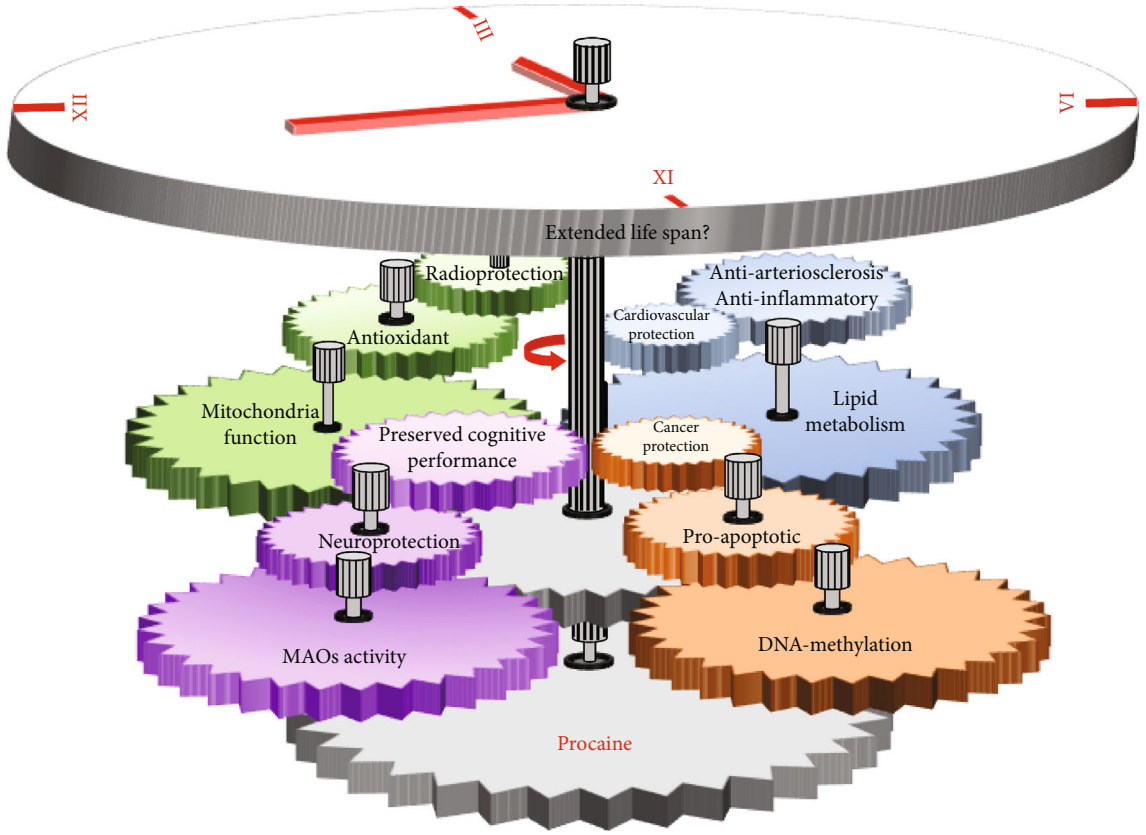

Figure 2: New biological and pharmacological effects of procaine—demonstrated within novel experimental approaches—which could acknowledge its consideration as a potential geroprotector candidate.

Recently, procaine was included among the potential "repurposed drugs" with promising results as an epigenetic modulator [129]. An important number of preclinical studies demonstrated the role of procaine as DNA-demethylating agent through the inhibition of DNA methyltransferases in normal [39, 139] and cancer cells [132], or through direct interaction with DNA [133]. In a variety of cancer cells, procaine is able to reactivate tumor suppressor genes, such as WIF-1 [130], and impair the survival and self-renewal of the malignant cells by inhibiting the activation of IL-6/STAT3 signaling [41]. Procaine also inhibits cancer cell proliferation and migration, enhancing apoptosis, through the inactivation of the ERK/MAPK/FAK and AKT/ERK pathways [134–139, 145] (Table 5).

In conclusion, beyond its well-known anesthetic action, procaine displays a variety of biological and pharmacological effects, functioning as an antioxidant, anti-inflammatory, cardioprotective, neuroprotective, radioprotective, cytoprotective, and demethylating agent. The beneficial effects on cellular functions and metabolism could designate procaine as a valuable candidate for the Geroprotectors (http://geroprotectors.org) database (Figure 2).

Future research approaches are likely to evaluate procaine's effects in animal and cellular experimental models, focusing on lifespan assessment, autophagy and proteasome regulation, replicative senescence—telomere length and telomerase activity, cell cycle regulation including aging-related pathways such as insulin/insulin-like growth factor 1 (IGF-

1)/phosphatidylinositol-3 kinase (PI3K)/AKT (Protein Kinase B) and the Forkhead box O (FOXO) transcription factors (FOXOs), as well as the energy sensing molecular apparatus comprising the mammalian target of rapamycin (mTOR), the adenosine monophosphate-activated protein kinase (AMPK), and the sirtuins. Only after extended investigation with novel experimental approaches, we will be able to fully understand the modulatory action of procaine in the mechanisms of aging and the etiology of chronic degenerative maladies. Following the in-depth comprehension of the fascinating multiples facets of procaine, a judicious use of procaine-based drugs could be employed in the prophylaxis and treatment of different metabolic and degenerative disorders commonly encountered in elderly patients, but which nowadays seem to affect younger and younger individuals.

Data Availability

Review article based on PubMed database.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Antioxidant and Signal-Modulating Effects of Brown Seaweed-Derived Compounds against Oxidative Stress-Associated Pathology

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The biological and therapeutic properties of seaweeds have already been well known. Several studies showed that among the various natural marine sources of antioxidants, seaweeds have become a potential source of antioxidants because of their bioactive compounds. Most of the metabolic diseases are caused by oxidative stress. It is very well known that antioxidants have a pivotal role in the treatment of those diseases. Recent researches have revealed the potential activity of seaweeds as complementary medicine, which have therapeutic properties for health and disease management. Among the seaweeds, brown seaweeds (Phaeophyta) and their derived bioactive substances showed excellent antioxidant properties than other seaweeds. This review focuses on brown seaweeds and their derived major bioactive compounds such as sulfated polysaccharide, polyphenol, carotenoid, and sterol antioxidant effects and molecular mechanisms in the case of the oxidative stress-originated disease. Antioxidants have a potential role in the modification of stress-induced signaling pathways along with the activation of the oxidative defensive pathways. This review would help to provide the basis for further studies to researchers on the potential antioxidant role in the field of medical health care and future drug development.

1. Introduction

Brown seaweeds are photosynthetic aquatic algae which belong to the domain of Eukarya, kingdom of Chromista, and class of Phaeophyta [1]. There are 1500 species of brown seaweeds all over the world [2]. In particular, in Asian countries, seaweed has been used as a traditional herbal medicine for the treatment of gastrointestinal problems, cough, boils, ulcers, asthma, cough, and headache as well as vegetables too [3]. Lately, several studies revealed that dietary seaweeds not only are a good source of carbohydrates, dietary fiber, proteins and peptides, vitamins, minerals, and fats but also contain a large concentration of functionally bioactive compounds such as carotenoids, polysaccharides, polyphenols, and sterols, which have potential antioxidant properties as well as antimicrobial, anticoagulant, antithrombotic, anti-inflammatory, antitumor, and antiviral properties for several diseases [4–6]. Therefore, nowadays, seaweeds have been paid attention to for the development of medicine, food, cosmetics, dietary supplements, fertilizer, and bioenergy [7].

Oxidative stress is widely involved in the development of many chronic diseases such as cardiovascular disease, neurodegenerative, cardiovascular, cancer, inflammation, diabetes, obesity, and aging and many of the elderly diseases [8–10]. Antioxidants are the only therapeutic molecules capable of blocking oxidative stress by their excellent reactive oxygen species (ROS) scavenging activity with low or no toxicity [11]. An antioxidant optimizes the human physiological function that helps to protect against disease or disease progression as well as maintains a healthy state [12]. Naturally, endogenous antioxidants are present in our body; the additional exogenous supplement can also be obtained from various natural sources and chemically synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) [13–15]. However, studies proved chemically synthesized antioxidants to be toxic and carcinogenic, whether the natural antioxidant is safe, more effective, and easily absorbed by the body [15]. The dietary antioxidants such as α-tocopherol, ascorbic acid, carotenoids, amino acids, peptides, proteins, flavonoids, and other phenolic compounds were found effective in the boosting of the antioxidant mechanism [16]. The marine world is a rich source of bioactive molecules. Among the various natural sources of antioxidants, marine seaweeds and their bioactive compounds are gaining worldwide attention [4, 17] in industry and drugs since 1980 [18]. Numerous evidences have shown that the brown seaweed-derived compounds are capable of improving the oxidative stress-induced diseases including neurodegenerative disease [19], cardiovascular-associated disorders [20], and obesity [21], as well as cancer protection [22]. But unfortunately, we do not have much information about the antioxidant effects and molecular mechanisms of brown seaweeds in oxidative stress diseases.

It has been reported that brown seaweeds have higher antioxidant properties comparatively than red and green seaweeds [23]. Brown seaweeds contain one of the most abundant pigment carotenoid compounds, fucoxanthin, and are estimated to contain around 10% of total carotenoids found in nature [24, 25]. Fucoxanthin has a great antioxidant activity as well as anti-inflammatory, antidiabetic, antiphotoaging, and neuroprotective properties [26]. Polyphenol compounds in brown algae such as phlorotannins are the unique and most dominant complex group of polymers named phloroglucinol (1,3, 5-trihydroxybenzene). It is mainly formed as secondary metabolites in the acetate-malonate pathway. Phlorotannin bioactive compounds exist as soluble compounds or cell-bound forms, mainly produced by brown seaweeds to protect themselves from herbivores and stress conditions, minimizing the oxidative damage caused by nutrient deprivation and ultraviolet radiation. Phlorotannins isolated from brown algae *Ecklonia cava*, one of the most abundant sources of polyphenolic compounds, have been revealed to have higher antioxidant activity *in vitro* and *in vivo* [6, 7, 20, 27]. Another bioactive compound of brown seaweeds is sulfated polysaccharides (SPs), which generally comprise a high content of major groups of sugar, e.g., fucose, galactose, uronic acid, and sulfate [28]. The antioxidant activities of SPs depend on their major sugar, degree of sulfating, low molecular weight, and glycosidic branching [29–31]. The major SPs found in brown seaweeds are fucoidan [32, 33]. Over the past decade, the antioxidant compound sterols called fucosterol have shown great antioxidant property as well as important contributions to human health and wellness [34].

This present review summarizes and discusses brown seaweeds and their major bioderived compound's role as an antioxidant in oxidative stress and their action in the management of disease-related signaling pathways. In Table 1, we show the antioxidant activity of brown seaweed-derived major compounds from the literature review and research articles.

2. Oxidative Stress, Antioxidant Defense, and Signal Transduction Pathway in the Cell

Generally, oxidative stress is the imbalance between the production of ROS and the body's own antioxidant defensive system [50]. ROS, usually generated through various extracellular and intracellular processes, are involved in cellular growth, differentiation, progression, and cell death as well as cell signaling [51, 52]. Excessive production of ROS causes oxidative stress and modifies the structure of the cellular macromolecules such as lipids, proteins, nucleic acids, and DNA. Consequently, the cellular and biological functions are inactivated with the modification of cellular signaling. Generally, under the physiological condition, ROS maintain the body's homeostasis by the regulation of several cell signaling pathways which are involved in cellular processes such as mitogen-activated protein kinase (MAPK), nuclear factor-kappa B (NF-κB), and phosphatidylinositol 3-kinase (PI3K) (Figure 1) as well as the defensive pathway nuclear factor erythroid 2–related factor 2 (Nrf2) signaling; therefore, ROS is considered as a secondary messenger for the activation of cellular signaling [50, 53–55]. Nrf2 is a redox-sensitive transcription factor that binds to antioxidant response elements (ARE) to regulate the expression of antioxidant enzymes that protect the cell against oxidative damage by inducing the

Table 1: Antioxidant activity of brown algal-derived compounds.

General category	Antioxidant compound	Antioxidant activity reported
Carotenoids	Fucoxanthin	[25, 35–39]
Polyphenols		
Phlorotannins	Phlorofucofuroeckol A, dieckol, phloroglucinol, eckol, 7 pholoroeckol, and 2-phloroeckol	[27, 40–43]
Sulfated polysaccharide	Fucoidans	[16, 32, 44–47]
Sterols	Fucosterol	[48, 49]

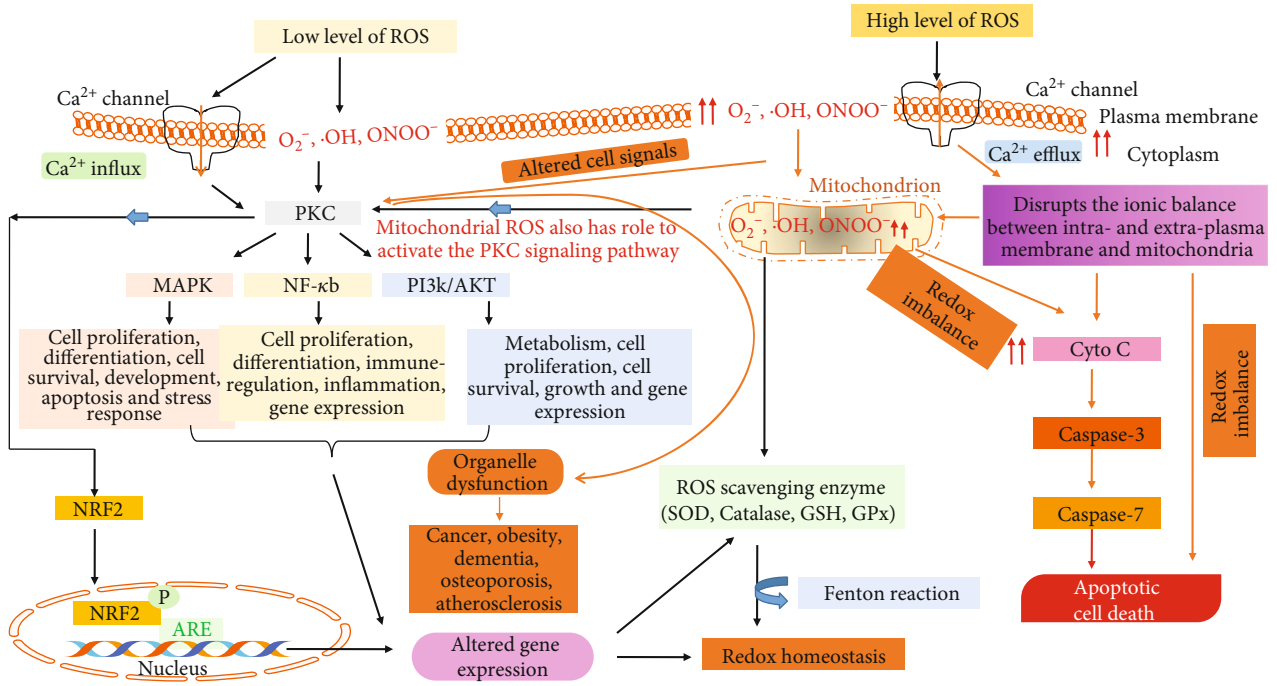

Figure 1: Redox homeostasis with cell signaling pathway under lower and higher levels of ROS.

antioxidant enzymes. Moreover, excessive ROS modulates the cellular antioxidant defense system that has eventually altered the normal physiological signal and switched to the apoptosis or cell death signals. Therefore, oxidative stress is to be involved in the development of many diseases including cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, schizophrenia, and chronic fatigue syndrome (Figure 2) [56–58].

In addition, ROS has a big role in balancing the intracellular and extracellular Ca^{2+} levels [54, 59]. Cellular Ca^{2+} content is also known as one of the most versatile signals for the activation of protein kinase C (PKC) signal transduction cascades which are involved in the control of cellular processes and functions, such as contraction, secretion, metabolism, gene expression, cell survival, and cell death as well as the maintenance of plasma membrane fluidity by proton motive force [60] (Figure 2). ROS with free radical groups such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), and peroxynitrite ($ONOO^-$) and the cellular Ca^{2+} level are well known to maintain the redox homeostasis and signaling events during normal physiological processes. Therefore, it is considered that the interaction between ROS and Ca^{2+} can be bidirectional [59–62]. Excessive and uncontrolled ROS signal or oxidative stress directly damages the plasma membrane fluidity and redox homeostasis [61, 62]. As a result, Ca^{2+} influx into the cytoplasm from the extracellular environment and disrupts the ion exchanging balance between the intra- and extra-cellular plasma membranes as well as mitochondria, which then leads to cell death or the apoptotic pathway by increasing the cytochrome c protein, which then in turn activates caspase 3 and caspase 9. The accelerated Ca^{2+} level disrupted the Ca^{2+} signal in the cellular cytoplasm, which dephosphorylates the protein and modulates the PKC signal transduction cascades, and is associated with many diseases and the aging process [60–62].

Primarily, antioxidants have the greatest defensive role in protecting the cell against oxidative damage [63]. Generally, antioxidants have a vital role in keeping optimal cellular functions and systemic health and well-being. However, under oxidative stress conditions, endogenous antioxidants in humans, although highly efficient, are not sufficient to protect the cell from the harmful effects of ROS [64]. Therefore, dietary antioxidants are required to maintain the optimal cellular defensive functions. The most efficient enzymatic

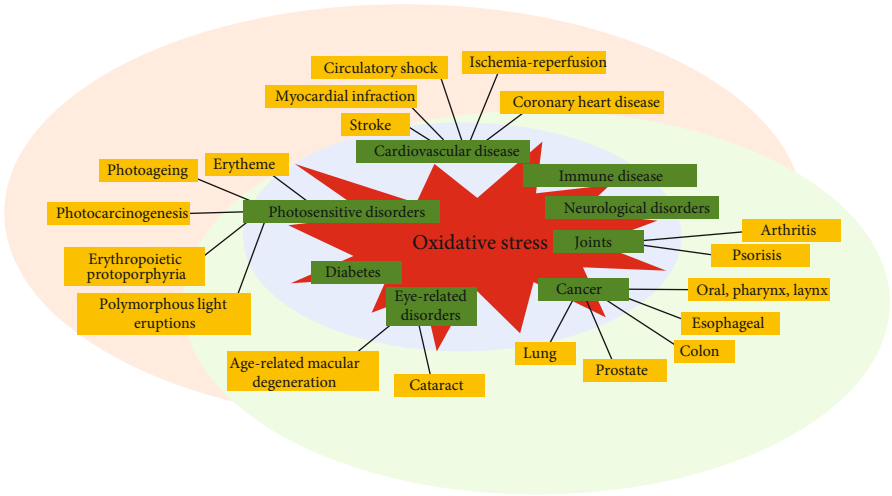

Figure 2: Examples of ROS and oxidative stress-induced diseases.

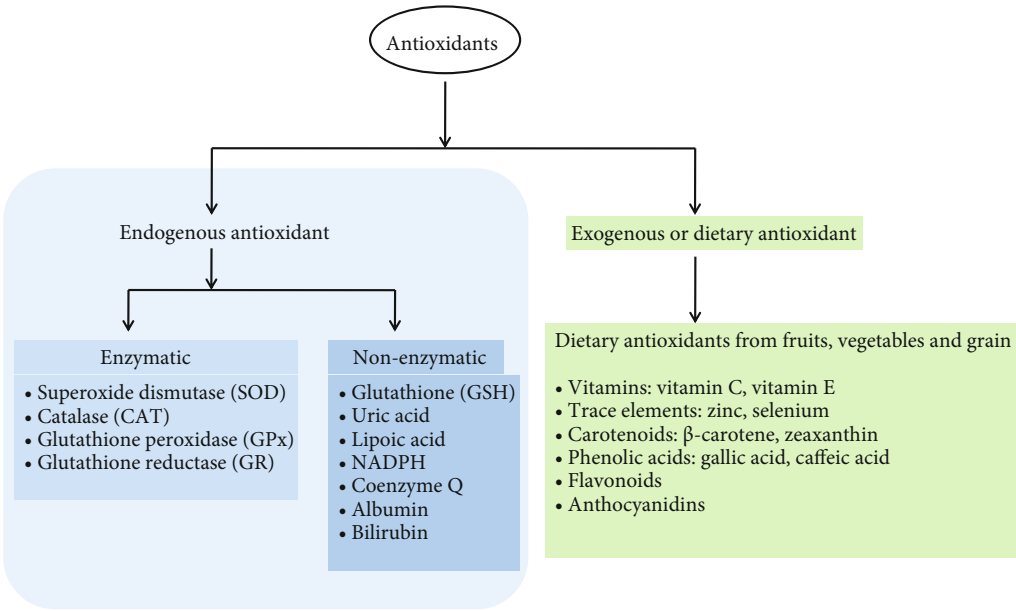

Figure 3: Classification of enzymatic and nonenzymatic antioxidants.

antioxidants contain glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), present in cellular cytoplasm and mitochondria. Nonenzymatic exogenous antioxidants are mainly derived in nature from photosynthetic organisms, fruits, vegetables, and plants and belong to different families such as vitamins E and C, thiol antioxidants (glutathione, thioredoxin, and lipoic acid), melatonin, carotenoids, and natural flavonoids [64–67] (Figure 3).

Antioxidants are considered as stable molecules that can donate an electron to a free radical and neutralize them, thereby scavenging the free radical and stopping the free radical from causing future damage [68]. Originally, antioxidants protect the cell from oxidative stress by applying one of the two mechanisms such as (a) the chain-breaking mechanism by which the primary antioxidant donates an electron to the free radical. (b) the second mechanism is the removal of ROS/reactive nitrogen species (RNS) initiators (secondary antioxidants) by quenching a chain-initiating catalyst. Antioxidants may provide their defensive action on biological systems at different levels including electron donation, metal ion chelation, radical scavenging, and repair or by gene expression regulation as well as prevent lipid peroxidation [69]. Moreover, antioxidants protect the cells by applying their strong multiphase efficacy system into the cell and cell membrane. It can strongly diffuse in both the aqueous and oil phases because of its polar and nonpolar paradox systems [70], known as a lipophilic, hydrophobic, and amphiphilic antioxidant (Figure 4). The efficacy of lipophilic antioxidants or polar antioxidants is to be more efficient than nonpolar or hydrophilic antioxidants. The lipophilic antioxidant can orient itself to the oil-water interface, where lipid peroxidation is induced whereas the hydrophilic antioxidant diffuses in the

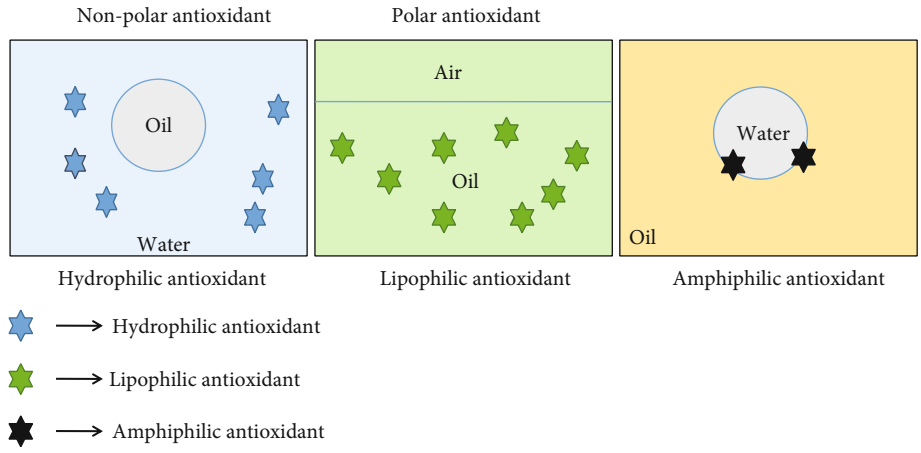

Figure 4: Efficacy of active antioxidants at different phases.

Table 2: Antioxidant efficacy of brown seaweeds compounds.

Antioxidant efficacy in the compound of brown seaweeds		
Lipophilic	Hydrophilic	Amphiphilic
Carotenoids [22, 78, 80]	Phlorotannins [22, 81, 82]	Sterols [22, 83, 84]
	Sulfated polysaccharide [22, 85]	

water phase, and therefore, it is less efficient [71]. Additionally, their activity also depends on the concentration of antioxidants, free radical types, and both the chemical structure and the reaction condition [69].

Nowadays, a large number of natural antioxidant compounds such as carotenoids, ascorbic acid, flavonoids, and phenolic compounds are derived from seaweed sources [22, 72–75]. The antioxidative efficiency of seaweeds is higher than that of plants and fruits [76–78]. It is already well reported that the brown seaweeds are the richest sources of these antioxidant compounds with a unique structure, which has a greater level of hydrophilic and lipophilic nature [14, 17, 79] (Table 2).

The redox equilibrium plays a pivotal role in the cell's physiological and pathological functions by balancing the ROS and antioxidant as well as ROS stability. ROS stability needs to activate or deactivate a variety of receptors, proteins, ions, and other signaling molecules [50, 53]. The excessive accumulation or depletion of ROS leads to instability of the redox balance that may influence many cellular signaling pathways and confers the cellular dysfunction as well as subsequently developing various pathologies [50]. To equilibrate the redox balance, antioxidants not only produce the antioxidant but also have a great ability to modulate the ROS sensitive cell signaling with the activation of the antioxidant responsive element ARE/Nrf2 pathway [54, 55]. However, numerous studies have already elucidated that the phytochemicals or natural compounds of antioxidants successively modulate the ROS-sensitive signaling pathway and restock the antioxidant into the cell through maintaining the redox balance [86]. The activation of such signaling pathways increased the expression of gene-encoding cytoprotective proteins, including phase II enzymes, antioxidant enzymes, growth factors, and proteins involved in the regulation of cellular energy metabolism. The activation of the Nrf2 pathway intersects with other intracellular signaling pathways such as the MAPK, PI3k/Akt, and NF-κB pathways [54, 55, 87] (Figure 5)

On the other hand, antioxidants actively reduce the cellular ROS which may help to balance the cellular redox balance as well as the intra- and extracellular cytoplasmic Ca^{2+}levels [87]. Antioxidants reduce the intracellular ROS level by balancing the ionic exchange between the plasma membrane and the cytoplasmic membrane as well as modulate the Ca^{2+} channel called IP inositol 1,4,5-trisphosphate receptor (IP3R) [54, 55, 59, 87]. As a result, the Ca^{2+} ion binds to the PKC enzyme and activates the PKC signaling cascades, as well as activates various signaling pathways and also triggers the release and translocation of Nrf2 from the cytosol to the nucleus, which is responsible for the expression of antioxidant genes, thus maintaining the cellular redox homeostasis [55, 59] (Figure 5).

Brown seaweeds are the largest type of macroalgae that belong to the phylum of Phaeophyta, which means "dusky plants." Naturally, it is brown or yellow-brown and found in temperate or arctic waters. Brown algae typically have a root-like structure called a "holdfast" to anchor the algae to a surface. There are about 1500-2000 species of brown algae worldwide [3]. The huge biodiversity of algae and the richness of physiological traits with their unique adaptive properties are considered as a potential target in the research area [88]. Most of the algae are oxygenic autotrophs; they can quickly and continuously adapt themselves to extreme environments by synthesizing their bioactive compounds with

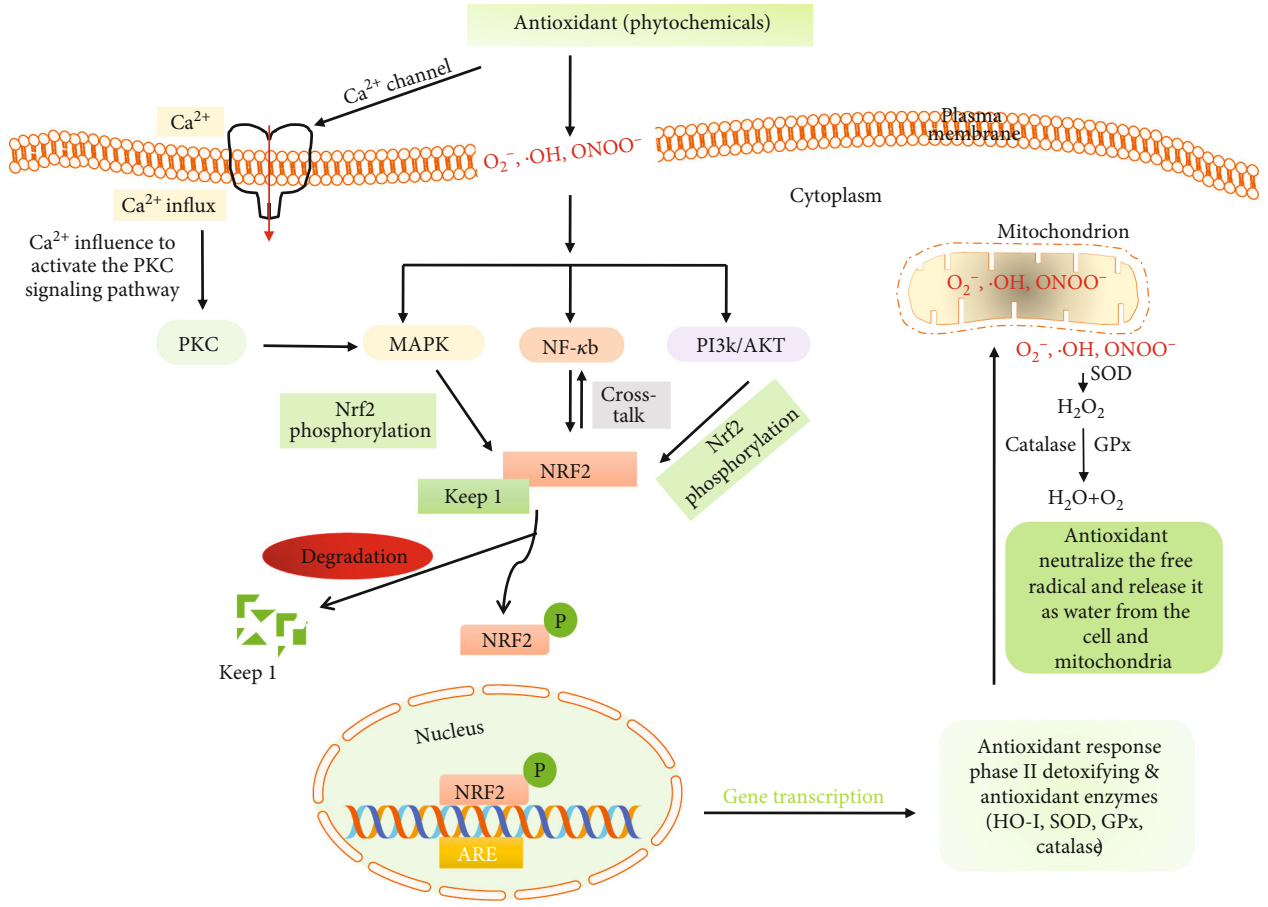

Figure 5: Antioxidant mechanism in the regulation of the Nrf2 signaling pathway with the crosstalk of the different signaling pathways.

protective functions, limiting or repairing potential damage from a harmful environment. These bioactive compounds act as primary and secondary sources of metabolites that provide the antioxidant and other bioactive functions as well as maintain their cellular homeostasis [3, 88]. It has been already proven in numerous research and clinical studies that the compound of brown algae species has antioxidative activity [23–25, 78, 81].

A large number of enzymatic and nonenzymatic antioxidants have been involved to detoxify the ROS and prevent the formation of highly reactive radicals such as hydroxyl radical (˙OH) [88]. Enzymatic antioxidants mainly produced in a cell are usually high molecular weight substances such as SOD, glutathione reductase (GR), CAT, and ascorbate peroxidase. Primarily, enzymatic antioxidants inactivate the ROS intermediates and detoxify the ROS by supplying the NADPH for proper functioning (Figure 6). On the other hand, the dietary substances known as exogenous antioxidants (such as carotenoids, flavonoids, phenolic compounds, and ascorbic acid) scavenge the free radicals to break down the chain reaction responsible for lipid peroxidation [66–69]. The compounds of brown algae such as carotenoids, polyphenols, sulfated polysaccharides, and fucosterols have belonged as exogenous antioxidants, which may apply two defensive actions to cope with ROS before they can damage the cellular deference mechanism [89, 90] such as

(1) The primary action is to break down the chain reaction, which results in free radicals becoming less reactive [66, 69]

(2) The second mechanism is scavenging activity against superoxide and hydroxyl radicals by chelating/deactivating metals. The metal-binding proteins (such as albumin, ferritin, and myoglobin) inactivate the transition metal ions (such as copper, iron, manganese, zinc, and selenium act to upregulate the antioxidant enzyme activities) that catalyze the production of free radicals, quenching/scavenging the singlet and triplet oxygen (highly toxic) and removing ROS [66, 91, 92].

3. Antioxidant Effects of Brown Seaweed-Derived Compounds

3.1. Carotenoids. Carotenoids are a family of pigmented compounds that are naturally synthesized by plants, algae, fungi, bacteria, and archaea [29]. Over 1100 naturally occurring carotenoids are produced from those sources but not from animals [93]. Carotenoids are hydrophobic highly conjugated 40-carbon (with up to 15 conjugated double bonds) molecules. About more than 500 species of natural carotenoids are known and categorized into two groups: primary carotenoids which have pure hydrocarbons (those without

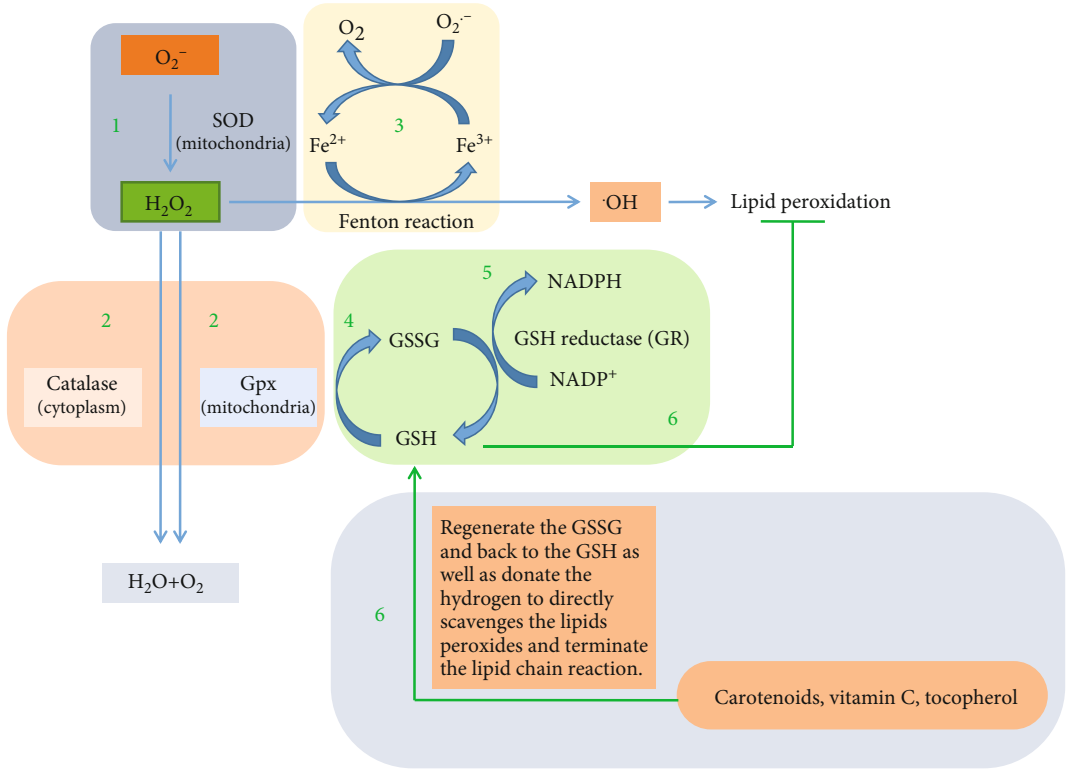

Figure 6: The enzymatic and nonenzymatic antioxidant protection mechanism. (1) Superoxide radical ($O_2^{\cdot-}$) is formed by a single-electron reduction of oxygen. In a reaction catalyzed by superoxide dismutase (Cu/ZnSOD or MnSOD), superoxide radical binds an electron, which leads to the formation of hydrogen peroxide (H_2O_2). (2) In the further reduction of hydrogen peroxide (H_2O_2) to water and oxygen by catalase (CAT) and glutathione peroxidase (GPx) enzymes. (3) In the Fenton reaction, the H_2O_2 is then transformed into hydroxyl radical ($HO^{\cdot}$) by catalyzing the transition metal, which is further participating in the free radical chain reactions. (4) H_2O_2 is reduced by glutathione (GSH) and produced glutathione disulfide (GSSH). (5) The glutathione disulfide is then reduced by glutathione reductase (GR) using the hydrogen of NADPH which is oxidized to $NADP^+$. (6) Besides, the nonenzymatic antioxidants such as carotenoids, vitamin C, and tocopherol support the regeneration of GSSG and back to GSH. Vitamin C, carotenoids, and tocopherol donate the hydrogen to free radicals that directly scavenges the free radical and terminates the lipid peroxidation chain reaction [66, 91, 92].

any oxygen molecule), e.g., α-carotene, β-carotene, and lutein, and secondary carotenoids which are carbon (C), hydrogen (H), oxygen-containing xanthophylls (e.g., fucoxanthin, astaxanthin, canthaxanthin, and echinenone) are generally produced during the photosynthesis of seaweeds after exposure to specific environmental stimuli such as light and osmotic shock [39, 94–96]. The brown seaweeds are the rich source of carotenoids, which have been reported as powerful antioxidants with numerous beneficial health effects (Figure 7) [96, 97]

Carotenoids (Crt) are very potent natural antioxidants. The antioxidant actions of carotenoids are based on their singlet oxygen quenching properties and the ability to trap free radicals, which mainly depend on the number of conjugated double bonds [98]. Furthermore, carotenoids can scavenge oxidizing free radicals via three primary reactions (Equations (1) to (3)), by its addition, electron transfer, addition, and hydrogen atom transfer.

The carotenoid antioxidant reactions are as follows: (i) electron transfer between the free radical ($R^{\cdot}$) and Crt, resulting in the formation of a Crt radical cation ($Crt^{\cdot}+$) (Equation (1)) or Crt radical anion ($Crt^{\cdot}-$) (Equation (2)); (ii) they can transfer the electrons forming a radical cation ($RCrt^{\cdot}$) (Equation (3)); and (iii) hydrogen atom transfer leading to a neutral Crt radical ($Crt^{\cdot}$) (Equation (4)) [38, 99].

$$R^{\cdot} + Crt \longrightarrow R- + Crt^{\cdot} + (1)[\text{Addition}] \quad (1)$$

$$R^{\cdot} + Crt \longrightarrow R+ + Crt^{\cdot} - (2)[\text{Addition}] \quad (2)$$

$$R^{\cdot} + Crt \longrightarrow RCrt^{\cdot}(3)[\text{Electron transfer}] \quad (3)$$

$$R^{\cdot} + Crt \longrightarrow RH + Crt^{\cdot}(4)[\text{Hydrogen ion transfer}] \quad (4)$$

Generally, the principal defense process in carotenoids is their lipophilic nature that allows them to penetrate through the cellular lipid bilayer membrane and cross to the blood-brain barrier, carrying out its biological function also in different regions of the human body, including the brain [39]. Because as a lipid-soluble molecule, carotenoids actively scavenge the lipid and aqueous phase radicals, mostly they are located in the apolar core of lipid membranes. Thus, carotenoids are associated with various types of membranes within the cell such as the outer cell membrane, but also the mitochondria and the nucleus. They also work in lysosomes. As a consequence, carotenoids play a major

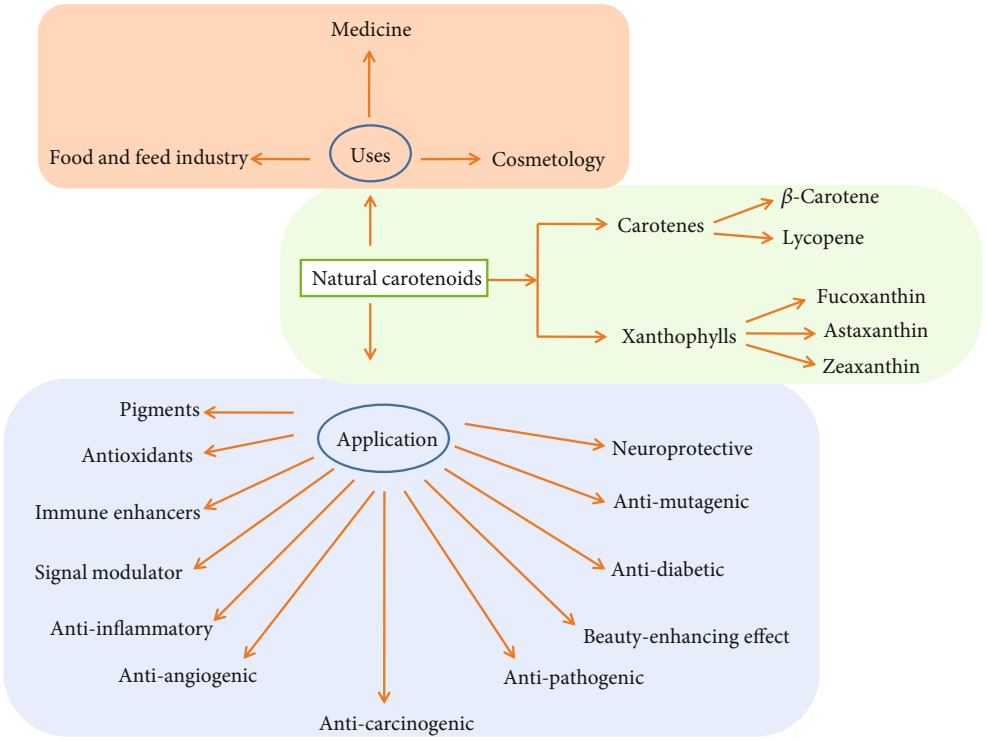

Figure 7: Distribution, biological functions, and application of natural carotenoids derived from brown seaweeds.

protecting role in the cell membranes and lipoproteins from damage by free radicals [100].

Several evidences suggested that carotenoids can act as redox agents in the case of oxidative stress due to their electrophile character and their conjugated double bonds that enhance the endogenous antioxidant Nrf2-Keap1 systems [101] which eventually help to maintain the redox homeostasis [80]. The redox homeostasis mainly depends on the nucleophile compounds, e.g., glutathione (GSH) and thiol (–SH) as well as their reductase enzymes GR, heme oxygenase-1 (HO-1), glutathione S-transferases (GSTs), and NAD(P)H quinine oxidoreductase 1 (NQO1). The GSH or –SH (thiol-) containing enzymes play a pivotal role in the free radical chain reaction that promotes to sustain the redox homeostasis (Figure 6) [102, 103]. Studies demonstrated that the carotenoids could increase the GSH level through the modulation of redox-sensitive –SH groups of Kelch-like ECH-associated protein 1 (Keap1). The Keap-Nrf2 signaling pathway plays an important role in the cellular defense against oxidative stress [104, 105]. Generally, under the normal physiological condition, Nrf2 is bound with their repressor protein Keap1 in the cytosol; which leads to its degradation by the ubiquitinylation process. Keap1 is a cysteine-rich protein, and its –SH residue conformation can be modified by different oxidants and electrophiles, thereby leading to the liberation of Nrf2 and transfer to the nucleus [80, 106]. At a higher oxidative stress condition, excessive ROS promotes the disassociation of Keap1 and Nrf2 either via the activation of PKC that assists to phosphorylate the Nrf2 or by oxidation of the cysteine residues of Keap1 that mainly govern the Keap1 activity [54–57, 107]. The carotenoids act as antioxidants through their electrophile C=C groups which conjugate with the aldehyde group (–CHO) of Keap1 modifying their thiol residues allowing the Nrf2 release into cytosol and translocation to the nucleus. Thus, Keap1 is inactivated, and newly synthesized Nrf2 binds to the ARE, which then leads to the expression of phase II enzymes and cytoprotective enzymes, e.g., GR, heme oxygenase-1 (HO-1), GSTs, NAD(P)H quinine oxidoreductase 1 (NQO1), SOD, and GPx [108] (Figure 8).

The scavenging function of carotenoids also has a signal-modulating role at the cellular signaling cascades such as the NF-κB, MAPK [109, 110], and PI3/Akt survival pathways as well the caspase pathway [111, 112]. Several studies suggest that carotenoids can act as modulators of redox-sensitive signaling cascades also able to progress the cell cycle through the direct modulation of cell cycle-related proteins as well as different signaling pathways which are usually involved in cell proliferation [108]. According to the studies, it can be hypothesized that β-carotene can modify the cellular redox status by changing the intracellular antioxidant status [112]. Studies showed that it can also delay the cell cycle G2/M phase by decreasing the expression of cyclin A in human colon adenocarcinoma cells [113] with increase of the antioxidant level which might help in decreasing the apoptotic protein as well as modifying the cellular growth [111]. It has been also reported that cell cycle progression and differentiation are dependent on the carotene dose, because it has a prooxidant role under high oxygen tension [113, 114]. Several indications suggested that carotenoids may also alter the expression of the apoptosis-related protein including the Bcl-2 and the caspase protein by a redox mechanism [111, 115]. Free radical species such as singlet oxygen [116] and nitric acid [117] have been

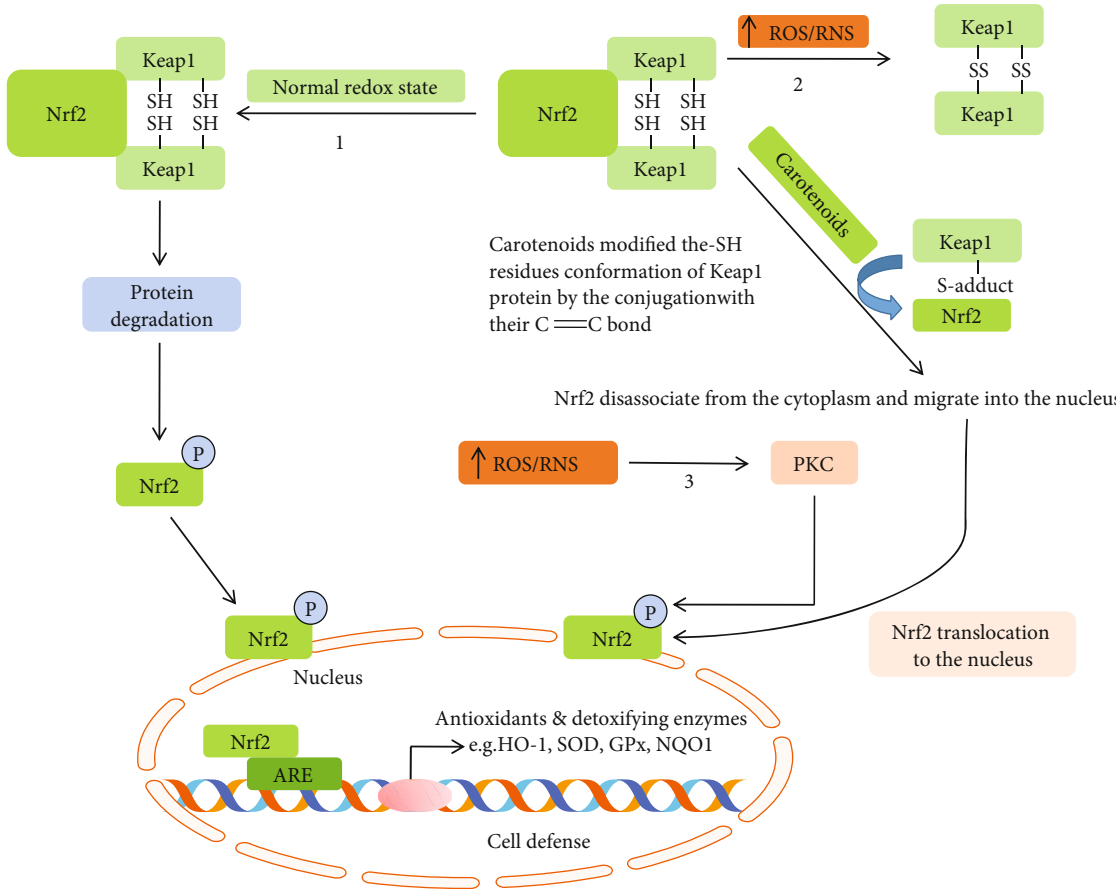

Figure 8: Electrophile activation of carotenoids of the Keap1-Nrf2 systems.

reported to activate the caspase 8, an important protein degrading enzyme involved in the apoptotic cascade [108], whereas studies found carotenoids can initiate the caspase 3 activities in several cell lines, mainly by interacting with a single complex located on the cell membrane and inducing caspase 8. It was also reported that carotenoids protect the colon cancer cell from apoptosis by decreasing the expression of antiapoptotic protein Bcl-2 [112]. Such effects are mainly strictly related to apoptosis induction and ROS production by the carotenoids. This finding interestingly demonstrated that carotenoids have a role in an antioxidant pathway, whereby this protein can prevent programmed cell death by decreasing the ROS formation and lipid peroxidation products [115]. *In vitro* studies observed that β-carotene may increase the expression of the proapoptotic protein Bax in U-937 cells and HUVEC cells [112].

The NF-κB pathway is generally thought to be a primary oxidative-response pathway [112]. Studies have shown that carotenoids can inhibit the oxidative stress-induced NF-κB pathway with the addition of their electrophile groups and the cysteine residues of IκB kinase IKK and NF-κB subunits (p65) [118]. It has been reported that β-carotene prevents the phosphorylation of ERK, JNK, and P38 in oxidative stress [119]. Recently, it has been reported that β-carotene may affect cellular growth through the modulation of redox-sensitive transcription factor AP-1 [112]. Carotenoids may also modulate the antioxidant response regulatory gene (ARE) and can alter the gene expressions of phase II enzymes (i.e., HO-1, GSTs, NQO1, SOD, and GPx), resulting in cellular redox homeostasis [112] (Figure 9).

Some of the studies indicate that carotenoids can also modulate oxidative stress-induced calcium signaling [120]. Carotenoids can decrease the calcium/calmodulin-dependent protein kinase IV (CAMKIV) enzyme by binding with the active site of CAMKIV; that form allows the cancer cell apoptosis [121]. CAMKIV is an enzyme belonging to the Ser/Thr kinase family. Generally, it plays a role in cell proliferation, migration, angiogenesis, and inhibition of apoptosis as well as calcium-dependent cell signaling. Elevated intracellular calcium ion concentration makes a complex form between $Ca^{2+/}$calmodulin, which induces the phosphorylation of the transcription factor and causes various types of cancer [122, 123].

One of the well-known carotenoids is fucoxanthin, considered as a potential antioxidant because of its unique chemical structure including an allelic bond (C=C), epoxide group, and hydroxyl group [124]. It is well-known, and unique structural carotenoid pigments are present in brown seaweed that have not been found in other carotenoids [35, 125]. Fucoxanthin accounts for around 10% of the total natural production of seaweed carotenoids [126], which shows strong antioxidative properties by the inhibition of intracellular ROS formation, DNA damage, and apoptosis as well

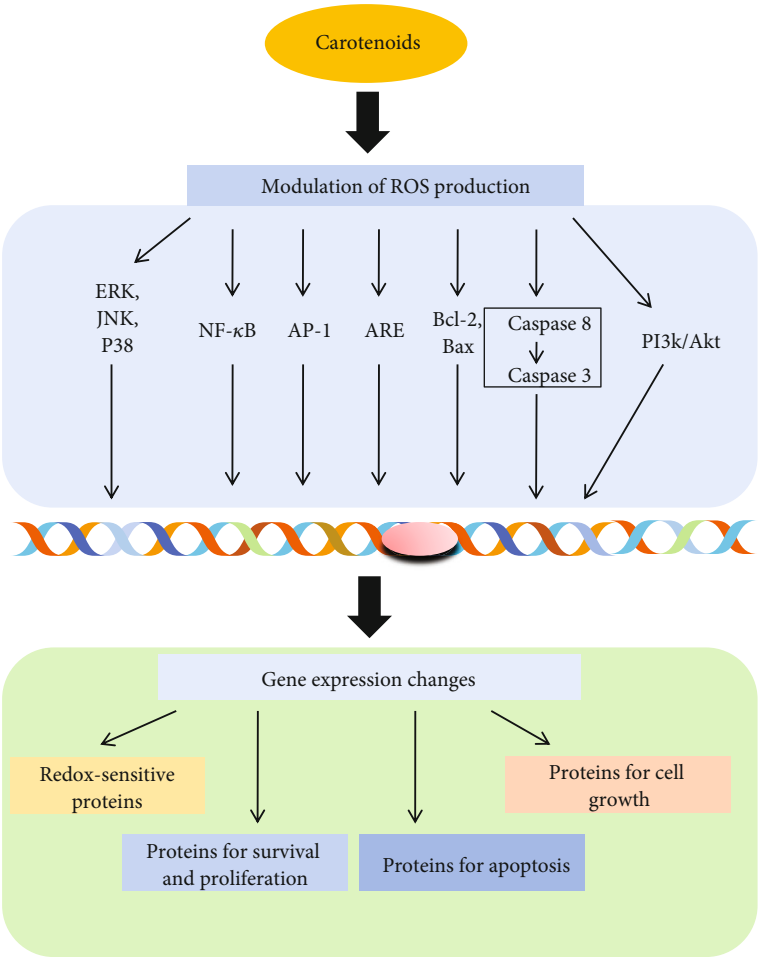

Figure 9: Scheme representing the multitude effects of carotenoids on the ROS-dependent signaling pathway and their related target protein gene expression. Carotenoid molecules may modulate the intracellular ROS production and can activate the redox-sensitive transcription factors or directly affect DNA damage which in turn may modify the gene expression.

as exhibiting strong enhanced cell viability against H_2O_2-induced oxidative stress [127]. Several studies have demonstrated their experiments that fucoxanthin is safe and nontoxic to cells even at repeated doses [128–131]. *In vivo* studies also have shown that a fucoxanthin diet elevated the antioxidant activities including CAT, SOD with the mRNA expression of Nrf2 and its target genes such as NQO1 [132]. It has an active role in the modulation of signaling pathways including MAPK [133], NF-κB [134], and apoptosis caspase pathways [135] as well as induces cell cycle arrest [136]. Recently, it was shown that fucoxanthin suppresses H_2O_2-induced inflammation and oxidative damage in microglial cells via the attenuation of the phosphorylation of the MAPK signaling pathway, as well as by the free radical scavenging capacity of fucoxanthin and its ability to regulate the endogenous antioxidant system [137] It can also exert the cytoprotective effects against H_2O_2-induced oxidative stress through the activation of the PI3K-dependent Nrf2-signaling pathway along with the expression of mRNA and protein relative cytoprotective gene LO2 cells [138]. Sangeetha and collaborators *in vivo* analyzed the properties of fucoxanthin compared with β-carotene [139]. These two carotenoids decreased lipid peroxidation as well as enhanced the CAT and GST activities, showing the protective effect against Na^+K^+-ATPase activity. However, fucoxanthin exhibited higher antioxidant and protection properties than β-carotene [39, 139]. Many studies suggested that fucoxanthin increases the intracellular cytosolic Ca^{2+} that triggers cell apoptosis through modulation of the cell cycle arrest [135, 136] (Figure 10).

3.2. Polyphenols and Phlorotannins. Algal polyphenols are a large and diverse class of secondary metabolites that consist of around 8000 naturally occurring compounds that possess vital biological functions including antioxidant and free radical scavenging properties [27, 140]. These biological properties mainly shared by this molecule are their phenol groups [27]. The antioxidant radical scavenging activity of these compounds generally precedes through the hydrogen atom transfer or electron transfer mechanisms [141–143]. The polyphenol compounds have one or more hydroxyl groups (-OH), which directly bond to a phenolic or aromatic hydrocarbon group, and the hydrogen atom transfer mechanism indicates the ability of the phenolic derivatives (ArOH) to

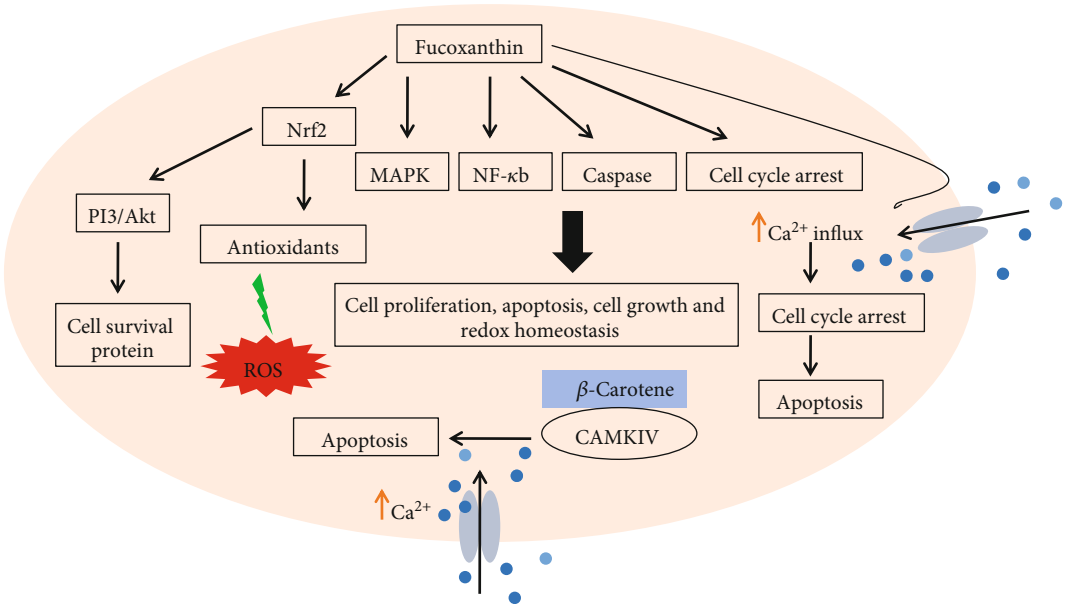

FIGURE 10: Carotenoids and fucoxanthin effects on the cellular calcium level along with the multitude effects of fucoxanthin on cellular signaling pathways. Fucoxanthin protects the cell from oxidative stress by modulating several cell signaling pathways.

transfer an -H atom from the phenolic -OH group [144]. Generally, the stability of the resulting phenolic radicals ($ArOH^{\cdot}$) governs the radical scavenging ability of these compounds [27].

$$R^{\cdot} + ArOH- = RH + ArO \cdot \quad (5)$$

In the electron transfer mechanisms, the ability to transfer an electron governs the radical scavenging activity of phenolic derivatives. The stability of these radical cations depends on their ionization potential. The lower potential value indicates the better stability of the electron [27].

$$R^{\cdot} + ArOH- = ArOH^{\cdot+} + R^{-} \quad (6)$$

Given the simplified reaction mechanism (Figure 11), phenols can react with free radical species ($R^{\cdot}$) giving phenolic radicals that undergo resonance stabilization within the molecule by delocalizing the unpaired electron within the aromatic ring leading to a stable intermediate (indicated by the resonance hybrid). This resonance stabilization stabilizes the radical scavenging ability of phenol compounds [27]

The antioxidant effect of polyphenols has also the ability to enhance the enzymatic activity of different enzymes, including catalase, glutathione peroxidase, and superoxide dismutase, by their potent free radical scavenging properties and their ability to interact with other molecular targets, as they are capable of activating the Nrf2/ARE pathway [145] (Figure 12). Among algal polyphenolic compounds, phlorotannins have also been shown to have a high antioxidant property because of their highly hydrophilic components and the presence of –OH groups that can form hydrogen bonds with water. They have a wide range of molecular sizes between 126 and 650 kDa and can occur in various concentrations (0.5-20%) in brown algae [27, 146]. Brown microalgae accumulate a variety of phloroglucinol-based polyphenols as phlorotannins of low, intermediate, and high molecular weight containing both phenyl and phenoxy units, whichever can also be linked with each other or other algae such as red algae. Based on this linkage, phlorotannins can be classified into 4 major subclasses: (a) fuchalos and phlorethols (phlorotannins with an ether linkage), (b) fucols (with a phenyl linkage, (c) fucopholoroethols (with an ether and phenyl linkage), and (d) eckols (with an ether and phenyl linkage) [146]. Pholorotannins isolated from *Ecklonia cava* and their compounds (phlorofucofuroeckol A, dieckol, phloroglucinol, eckol, 7-pholoroeckol, 2-phloroeckol, and fucodipholoroethol G) are one of the most abundant sources of polyphenolic that have been mostly reported of biological activities including anti-inflammatory, antidiabetic, anticancer, antimicrobial, and antiviral, whereas most of them are focused on antioxidant activity [20, 146, 147]. Many researchers have shown that the eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol have shown potent inhibition of phospholipid peroxidation at 1 μM in a liposome system [147, 148], and these phlorotannins have significant radical scavenging activities against superoxide and DPPH radicals effectively compared with ascorbic acid and α-tocopherol [09]. There are several pieces of research that have shown that *Ecklonia cava*-derived phlorotannins and their bioactive compounds can modulate the ROS-mediated intracellular signaling pathway as well as gene expression and inhibit the enzymes [20, 145, 149, 150], which eventually protect the cellular damage from oxidative stress and apoptosis [20]. Studies also showed the phlorotannins may also protect the cancer cell by the inhibition of ROS-generating MMP-2 and MMP-9 via the NF-

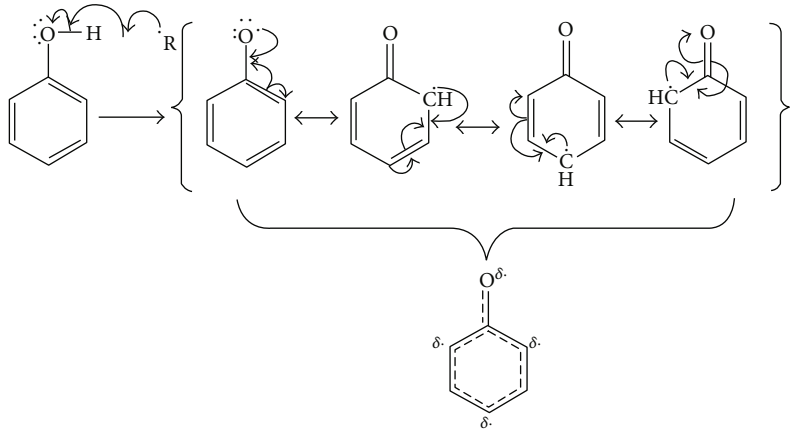

Figure 11: The proposed reaction between phenol and a free radical, which undergoes resonance stabilization; indicates the radical scavenging ability of compounds (i.e., curved half-headed arrows represent the transfer of a single electron).

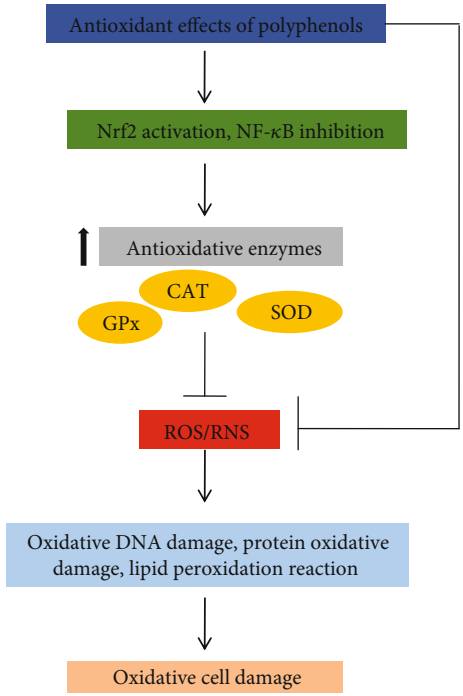

Figure 12: Potential mechanisms of polyphenols to the protection of the cell against oxidative stress.

κB pathway [151]. It has been reviewed that phlorotannins can block the Ca^{2+} channel as well as reduce the Ca^{2+} influx which are stimulated by ROS (Figure 13) [20]. Due to several profound capabilities of *E. cava*, derived compounds are interesting to use as functional ingredients in pharmaceuticals and cosmeceuticals [147, 152, 153]

3.3. Sulfated Polysaccharides. Marine SPs are polymeric carbohydrates with large hydrophilic molecules. They have a fundamental role in living marine organisms such as energy storage and protection or as a structural molecule, which are mainly produced by photosynthesis [85]. The algal polysaccharides possess potent bioactivities because of their unique physicochemical properties, such as high content of fucose, galactose, uronic acid, and sulfate [154]. Microalgae can accumulate carbohydrates to more than 50% of their dry weight, due to the high photoconversion efficiency of the photosynthetic process [155]. These compounds are present in high concentrations and vast diversity in microalgae [156]. A vast number of researchers have reported that the SPs and its compounds could alleviate oxidative stress-mediated diseases, such as liver injury, diabetes, obesity, neurodegenerative disease colitis, and cancer [155–161]. This effect could be explained by three distinct mechanisms, including scavenging the ROS and regulating the antioxidant system or oxidative stress-mediated signaling pathways and also implying the complicated interactions of marine-derived antioxidant polysaccharides in reducing the oxidative stress [46, 47] (Figure 14). The antioxidant activity of SPs depends on their structural property, such as the degree of sulfating, their molecular weight, types of the major sugars, and glycosidic branching [46, 162]. The low molecular weight SPs have been shown to be potent antioxidants rather than the high molecular weight SPs [163]. It has been also reported that low molecular weight (~30 kDa) SPs can promote cell proliferation [164] as well as cell protection from the oxidative stress-induced apoptosis, which are directly and indirectly related to their antioxidant properties [46, 165]. Currently, SP has become a hot research area in the field of regenerative medicine and tissue engineering application due to its unique structure and specific features as antioxidants, antitumors, immunomodulatory, inflammation, anticoagulant, antiviral, antiprotozoal, and antibacterial [46, 47, 162, 165].

Among the three types of SPs extracted from seaweeds, the brown seaweeds are the richest source of antioxidants [46, 47]. The major bioactive SP extracted from brown seaweeds is fucan which is usually defined as fucoidan; it is water-soluble and composed of significant quantities of L-fructose and sulfated ester groups accompanied with a little percentage of other monosaccharides, e.g., arabinose, xylose, glucose, galactose, and mannose. These constitutes of polysaccharides are known to be one of the major components of the brown algae cell wall that protects the cell in stress or low tide environments [46, 165].

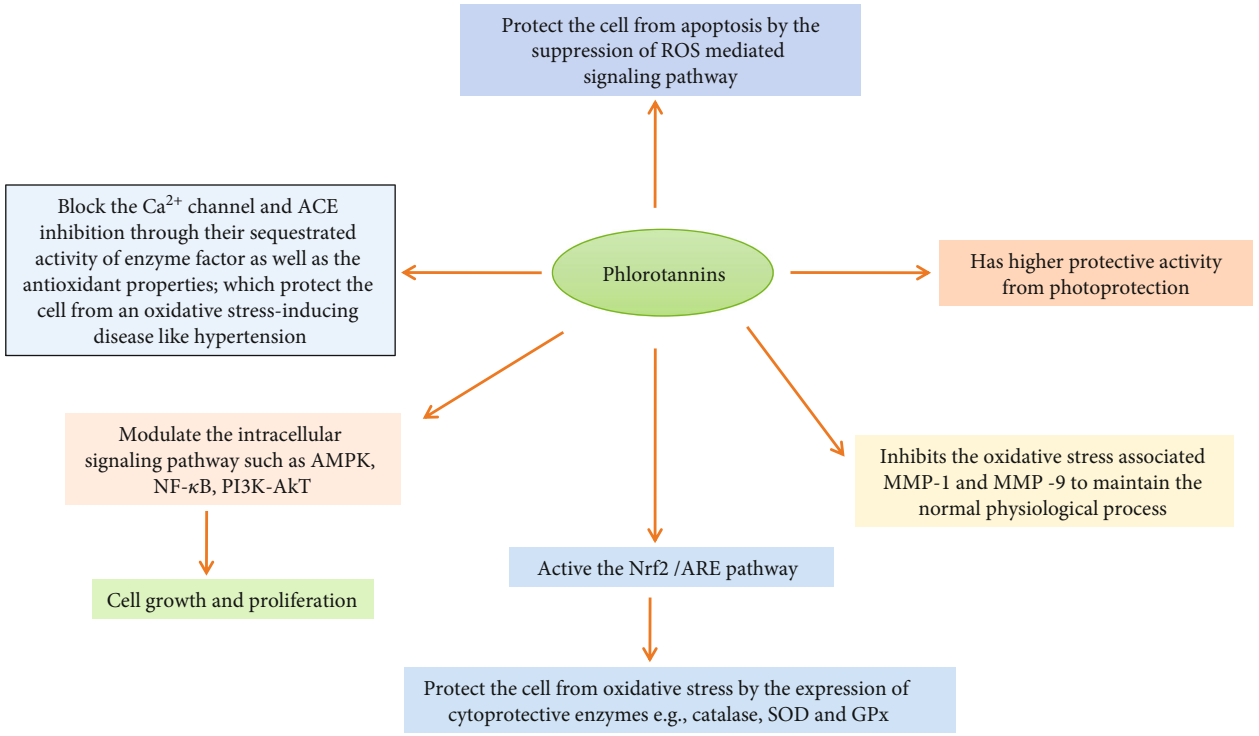

Figure 13: The biological role of phlorotannins on the function of the cell signaling pathway.

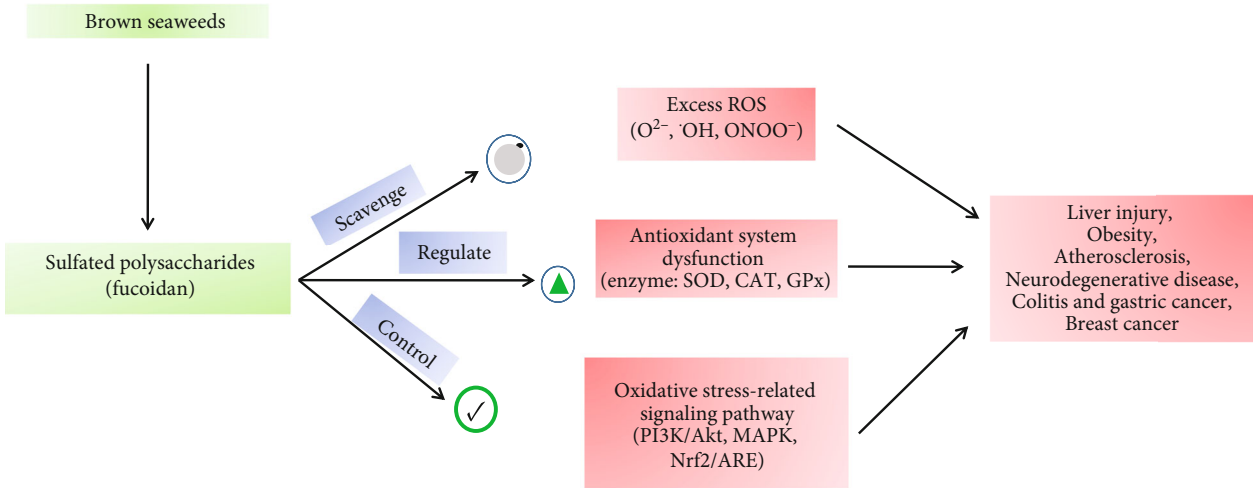

Figure 14: Overview of sulfated polysaccharide (fucoidan) derived from brown seaweeds in alleviating oxidative stress-mediated disease.

Numerous *in vivo* and *in vitro* studies have revealed that fucoidan derived from brown algae can alleviate body damage from oxidative stress through the regulation of the antioxidant system in the body [45, 165, 166]. The excellent antioxidant activity of fucoidan is mainly dependent on their desulfation or oversulfation that allows the development of derivatives of fucoidans [46, 165, 166]. The DPPH free radical scavenging assay is widely used to evaluate the *in vitro* antioxidant activity [167]. Polysaccharides provide the hydrogen or electrons to DPPH free radicals to form stable molecules (DPPH-H) [168]. The electron-withdrawing group of polysaccharides and the specific structures activate the hydrogen atoms on sugar residues [169]. Many studies have already found that sulfated polysaccharides from brown algae have strong DPPH free radical scavenging activity and reduction ability. Furthermore, SPs also have strong ferrous ion-chelating and reducing power activities [170, 171]. Many studies have revealed that fucoidan inhibits oxidative stress by downregulating the malondialdehyde (MDA) level and upregulating the SOD level [172]. Moreover, studies have documented that fucoidan extract can reduce the levels of lipid peroxidation markers MDA and TBARS in alcoholic rats and increase the levels of the nonenzymatic antioxidant GSH and the enzymatic antioxidant SOD, CAT, and GPx in the liver, ultimately reducing the oxidative damage caused by

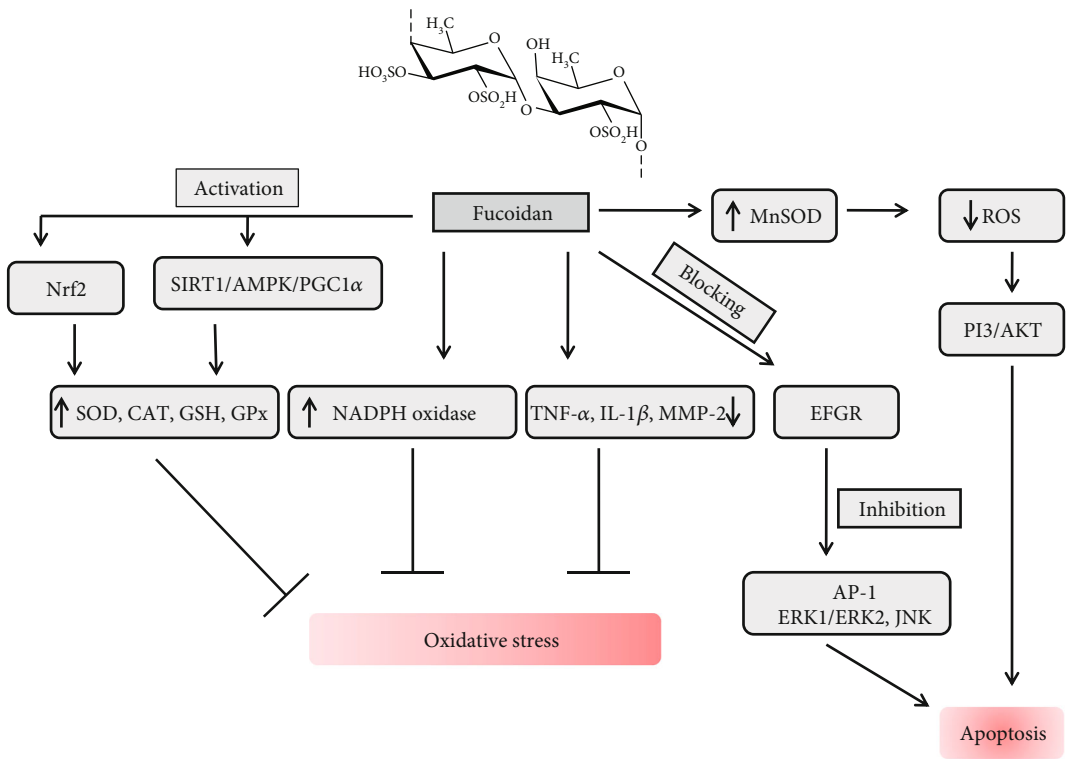

Figure 15: Fucoidan prevents oxidative stress and cell apoptosis by regulating the antioxidant system and signal-modulating pathway.

alcohol. In addition, fucoidan also reduces the mRNA expression of TNF-α, IL-1β, and MMP-2 to inhibit the production of ROS in the liver, thus alleviating the development of nonalcoholic fatty liver disease [173]. Similarly, fucoidans inhibit the proliferation and induced apoptosis through the downregulation of the PI3K/AKT pathway as well as the reduction of Bcl-2 and Bcl-xl and the rise of the expression of Bax, caspase 3, and caspase 9 activation [174]. It can also inhibit the epidermal growth factor (EGF) signaling in several cancer cell types, by preventing binding their receptor site that promotes to block the activation of activator protein-1 (AP-1) which also diminishes the transactivation activity of ERK1/ERK2 and JNK signaling protein [175]. Another study also showed that fucoidan can inhibit apoptosis and improve the cognitive ability in Alzheimer's disease model mice by upregulating the expression of the apoptosis-inhibiting proteins by activating the SOD activity and increasing the GSH levels [176]. Recently, a study found that fucoidan can improve atherosclerotic cardiovascular disease by reducing the production of ROS by inhibiting NADPH oxidase (Figure 15) [177]. Besides, fucoidan can also inhibit ROS production through the regulation of the antioxidant defense system, thereby alleviating oxidative stress-related diseases. A study revealed that low molecular weight fucoidan regulates the activity of SOD and CAT by activating the SIRT1/AMPK/PGC1α signaling pathway, inhibiting the superoxide production and the lipid peroxidation, reducing TNF-α and transcription factor NF-κB, and then inhibiting oxidative stress in the liver (Figure 15) [164]. Furthermore, fucoidan significantly increased the expression of MnSOD and decreased the ROS level through the PI3/AKT pathway and finally enhanced the survival and angiogenesis of mesenchymal stem cells in the ischemia model [178]. Interestingly, it was reported that it could upregulate the expression of HO-1 and SOD-1 genes by activating Nrf2 and then attenuate the oxidative stress in HaCaT cells [163]. Research also showed that the *Turbinaria ornata* protects the human carcinoma cell through the modulation of PPAR gamma, NF-κB, and oxidative stress [179]. Another study also proved that the *Turbinaria ornata* protects the hepatic injury from azoxymethane-induced oxidative stress by exerting multiple pathways including the abolishment of inflammation and oxidative damage and the activation of PPAR gamma with increase of the antioxidant enzymes like SOD and GPx activities [180]. Thus, we could be review by all of these studies that fucoidan derived from brown algae not only has significant antioxidant activity but also modulates the oxidative stress-mediated diseases by regulating the antioxidant defense systems and the oxidative stress-related signaling pathway in cellular and experimental animal models [22].

3.4. Sterols. There are abundant sterols in brown seaweeds. These compounds occur in the free form, esterified with fatty acids, or are involved in glycosylated conjugates [49, 181]. Sterols are amphipathic and triterpene compounds that can reach about 5.1% of the total biomass in the microalgae [181, 182]. Most of them contain 28 or 29 carbons and 1 or 2 carbon-carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain

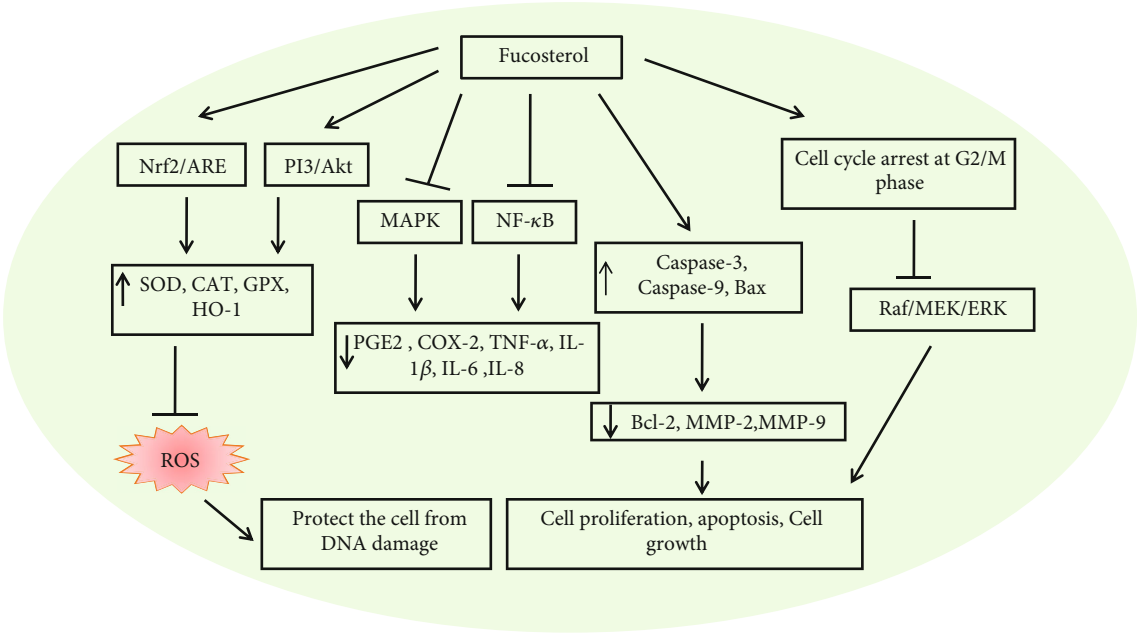

Figure 16: Overview of the biological role of fucosterol derived from brown seaweeds in alleviating the oxidative stress-mediated signaling pathway.

[181]. Moreover, fucosterol displays a high diversity of the unique compounds of phytosterols, such as brassicasterol, sitosterol, and stigmasterol [182, 183]. Some species contain a mixture of ten or even more phytosterols [183]. Sterol composition varies depending on the algae strain and can be modulated by light intensity, temperature, or growth phase [83]. Sterols have received much attention in the last few years because of their cholesterol-lowering properties [181]. Fucosterol is structural and functionally similar to cholesterol; however, it contains an alkyl; fucosterol, a phytosterol found in brown seaweeds, is well recognized for its various beneficial biological activities [49].

Studies showed that fucosterol elevates the activities of free radical scavenging enzymes such as SOD, CAT, and GPx against hydrogen peroxide. It can also restore the SOD and GPx cellular defensive enzymes which help to prevent cell membrane oxidation [48]. It can also suppress the nitric oxide (NO) and ROS generation through the suppression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expression because increasing iNOS and COX-2 is responsible for inflammatory disease [49]. Moreover, fucosterol exhibits inhibitory activity against acetyl- and butyryl-cholinesterases (AChE and BChE, respectively) [184, 185] and β-secretase enzyme which is responsible for Alzheimer's disease, although this enzyme production level is mainly associated with increasing the oxidative stress [186].

In the study of pharmacology and *in silico* analysis, it was revealed that the fucosterol action could intervene in the disease progression through modulating the biological processes as well as the signaling pathway, such as cytokine-mediated signaling pathway, apoptotic process, transcription regulation, inflammatory response, aging, response to lipopolysaccharide, NF-κB activity, and also cellular response to ROS, which are closely associated with the disease pathophysiology. Studies showed that fucosterol could pass through the cell membrane to reach directly to various intracellular targets. The pharmacological network data reported that fucosterol showed a close association with the target proteins of many crucial pathways at the molecular and cellular levels [187]. Another report demonstrated that fucosterol attenuates the oxidative stress through the upregulation of antioxidant enzymes such as GPX1, SOD, CAT, and HO-1 via Nrf2/ARE and PI3/Akt signaling activation [49, 188].

Fucosterol treatment can inhibit the oxidative stress-induced MAPK and NF-κB signaling pathway along with reducing the levels of inflammatory mediators PGE2 and COX-2, as well as proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 [188]. Studies represented an excellent role of fucosterol in cancer cell proliferation, cell cycle, and apoptosis [189]. It was found that fucosterol causes the arrest of the cancer cells at the stage of the G2/M phase that prevents the cancer cells to enter the mitosis stage which can prevent cell growth and proliferation [190]. The Raf/MEK/ERK signaling pathway contributes to cancer cell proliferation. It also showed that fucosterol induced cell cycle arrest, via the inhibition of Raf/MEK/ERK signaling pathways that ultimately prevents cell proliferation [191]. Fucosterol can induce cancer cell apoptosis by increasing the expression of cleaved caspase 3, caspase 9, and Bax and decreasing the expression of Bcl-2, MMP-2, and MMP-9 [189, 192] (Figure 16). Research showed that the species of fucosterol named *Padina pavonica* exerted an excellent antidiabetic and antioxidant effect via the activation of PPARγ mechanisms [193]. Fucosterol treatment can protect the cell from DNA damage after oxidative stress [188]

4. Conclusion

The research on brown seaweeds and their bioactive compounds' biological spectrum has been widely raised in recent years. Brown seaweeds and their derived compounds such as phlorotannins, SPs, fucosterol, and carotenoid pigments including fucoxanthin radical scavenging property are now clinically emphasized as a novel therapeutic compound. Antioxidants not only scavenge the free radical but also have numerous roles in the signal transduction pathway. From this point of view, we have attempted to gather plenty of information on how brown seaweeds and their antioxidant compounds modulate the oxidative stress signaling pathway as well as regulate the antioxidant system. However, the mechanisms of seaweeds are still obscure in the case of stress-causing disease. Thus, more studies should be focused on the investigation of activation and modulation as well as specific effects on the signaling pathway. The potentiality and structural features of the seaweed compound can have different effects on the cell. Additionally, long-term toxicity and drug delivery approaches should be evaluated.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

All authors contributed to the article and approved the submitted version.

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Research Article

UNC5B Promotes Vascular Endothelial Cell Senescence via the ROS-Mediated P53 Pathway

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Vascular endothelial cell senescence is involved in human aging and age-related vascular disorders. Guidance receptor UNC5B is implicated in oxidative stress and angiogenesis. Nonetheless, little is known about the role of UNC5B in endothelial cell senescence. Here, we cultured primary human umbilical vein endothelial cells to young and senescent phases. Subsequently, the expression of UNC5B was identified in replicative senescent cells, and then, its effect on endothelial cell senescence was confirmed by UNC5B-overexpressing lentiviral vectors and RNA interference. Overexpression of UNC5B in young endothelial cells significantly increased senescence-associated β-galactosidase-positive cells, upregulated the mRNAs expression of the senescence-associated secretory phenotype genes, reduced total cell number, and inhibited the potential for cell proliferation. Furthermore, overexpression of UNC5B promoted the generation of intracellular reactive oxygen species (ROS) and activated the P53 pathway. Besides, overexpression of UNC5B disturbed endothelial function by inhibiting cell migration and tube formation. Nevertheless, silencing UNC5B generated conflicting outcomes. Blocking ROS production or inhibiting the function of P53 rescued endothelial cell senescence induced by UNC5B. These findings suggest that UNC5B promotes endothelial cell senescence, potentially by activating the ROS-P53 pathway. Therefore, inhibiting UNC5B might reduce endothelial cell senescence and hinder age-related vascular disorders.

1. Introduction

Cardiovascular and cerebrovascular disorders immensely contribute to the global health and economic burden [1]. Epidemiological studies reveal that advanced age is a major risk factor for cardiovascular and cerebrovascular diseases [2, 3]. Moreover, emerging evidence indicates that shared molecular mechanisms of vascular aging underlie the pathogenesis of age-related macrovascular and microvascular diseases [3]. Vascular endothelial cells residing in the innermost layer of vascular tissue occupy a crucial position in vascular health. Therefore, establishing the molecular mechanisms underlying endothelial cell senescence is necessary.

Similar to most cellular senescence, endothelial cell senescence program is triggered by extracellular and intracellular stresses like telomere shortening, oxidative stress, DNA damage, and epigenetic changes [4]. These stressors could be interrelated and engage various downstream effector pathways but ultimately activate P53, P16, or both [5, 6]. Among them, oxidative stress, triggered by the accumulation of the reactive oxygen species (ROS), is a primary mechanism underlying the cellular senescence process [7] and promotes age-associated endothelial dysfunction [8]. Despite moderate ROS levels being essential for normal functions in cells, overproduction of ROS causes deleterious effects, including DNA damage. In response to DNA damage, the P53-dependent pathway is activated causing a P21-dependent cell cycle arrest [9]. Specifically, significant evidence demonstrates that senescent endothelial cells are dysfunctional exhibiting age-related impairment of angiogenesis [10–12]. Age-related impairment of angiogenesis is considered to be one of the primary factors causing increased morbidity and mortality of vascular disease [13]. Accordingly, understanding the mechanisms of oxidative stress in age-related endothelial cell

senescence can help to address the increased risk of age-related vascular disorders.

Guidance receptors have been demonstrated to not only participate in axonal sprouting in the nervous system but also take part in the formation and remolding of the vascular systems [14]. Moreover, guidance receptors regulate the progression of neurological and cardiovascular disorders [14]. Transmembrane receptor UNC5B is one of the vascular-specific axon guidance receptors whose expression is detected in various tissues, specifically in endothelial cell clusters [15–18]. Dysregulation of UNC5B expression in macrophages is implicated in atherosclerosis development and plaque stability [19]. Nonspecific repression of UNC5B significantly attenuated myocardial ischemia-reperfusion injury [18]. As such, UNC5B might be involved in the development of age-related disease. Although UNC5B has been demonstrated to be related to angiogenesis in endothelial cells [20], their relationship with aging remains unreported. A recent study has shown that UNC5B might be implicated in the generation of intracellular ROS induced by ultraviolet-B irradiation exposure [21]. However, the regulatory role of UNC5B alone in oxidative stress and other biological processes is unclear. Besides, UNC5B was also reported to act as a tumor suppressor and mediate P53-dependent apoptosis [22–24]. Since senescence is conventionally a potent tumor suppressor mechanism, UNC5B could contribute to cellular senescence.

The parabiotic model supports the idea that transferable factors in blood rescue age-related degenerative phenotypes [25–28]. Our preliminary research indicated that UNC5B participated in restoring endothelial cell senescence and age-related endothelial dysfunction with young human plasm. To evaluate our hypothesis, i.e., UNC5B regulates endothelial cell senescence, a prototypical model of replicative cellular senescence was used in primary human umbilical vein endothelial cells (HUVECs). We demonstrated that UNC5B was induced in replicative senescent endothelial cells and overexpression of UNC5B accelerated senescence. Thereafter, since oxidative stress is a primary factor of cell senescence, and P53 modulation potentially influencing both senescence and apoptosis, this work investigated if senescence triggered by UNC5B could be attributed to the ROS-P53 pathway. Eventually, we examined the impacts of UNC5B on age-related vascular function.

2. Materials and Methods

2.1. Isolation and Culture of Endothelial Cells. Primary HUVECs were obtained from five different human umbilical cords, as previously described [29]. The collection of human umbilical cords was approved by the Ethics Committee for Human Experimentation of Tongji Hospital. Isolated HUVECs were cultured in complete Medium 199 supplemented with 10% FBS (Biological Industries, Israel) and Low Serum Growth Supplement (Gibco, USA). HUVECs were passed with 0.05% trypsin-EDTA (Gibco, USA) at intervals of every 2-3 days. The population doubling level (PDL) was obtained based on previously described formulas [30]. Replicative senescent cells were generated by consecutively passaging the young HUVECs until the cell proliferation was nearly arrested, which was approximately at PDL 29 but varied from one individual to another. Young and senescent cells used in the experiments were cells from PDL 5 to 13 and PDL 25 to 33, respectively.

2.2. Senescence-Associated-β-Galactosidase (SA-β-gal) Assay. The SA-β-gal activity was measured using Senescence Cells Histochemical Staining Kit (Sigma, USA) based on the manufacturer's instructions. Briefly, cells were incubated in the 1X Fixation Buffer at room temperature for 6-7 min then stained with the Staining Mixture at 37°C without CO_2 overnight. Subsequently, cells were observed and visualized under a light microscope. The blue-stained cells and the total number of cells were counted using ImageJ (NIH, USA).

2.3. RNA Extraction and Quantitative RT-PCR (qPCR) Analysis. Total RNA was purified by RNA purification kit (Magen, China) and reverse-transcribed using the ReverTra Ace® qPCR RT kit (TOYOBO, Japan) following the manufacturer's instructions. SYBR® Green Realtime PCR Master Mix (TOYOBO, Japan) was applied in determining the mRNA levels of different genes using the primers listed in Table 1 on an ABI Step One Plus (Applied Biosystems, USA). GAPDH was used as an endogenous control.

2.4. Western Blot. Total protein was lysed in RIPA buffer (Boster, China) containing a protease inhibitor cocktail (Boster, China). Protein lysates were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred onto polypropylene difluoride membranes (Millipore, USA). The membranes were blocked in 5% non-fat milk at room temperature for 1 hour and incubated with the following antibodies: UNC5B (1 : 1000; Cell Signaling Technology, USA), P53 (1 : 1000; R&D, USA), P21 (1 : 500; Santa Cruz, USA), and GAPDH (1 : 5000; Proteintech, China) at 4°C overnight. After washing 3 times in 1X TBST (Tris-buffered saline containing 0.1% Tween 20), the membranes were incubated with respective secondary antibodies conjugated with horseradish peroxidase (1 : 5000; Promotor, China) at room temperature for 1 hour. Eventually, the membranes were rewashed three times with TBST and visualized using an enhanced chemiluminescence kit (Beyotime, China).

2.5. RNA Interfering. Small interfering RNA- (siRNA-) targeting UNC5B and negative control (NC) were designed and synthesized by RiboBio (RiboBio, China). Replicative senescent HUVECs were transfected with the siRNAs using Lipofectamine™ 3000 (Thermo Fisher, USA). Cells were seeded in a complete medium approximately 16 hours before transfection. Subsequently, siRNA mixed with Lipofectamine™ 3000 was added to the cells with a fresh 2% FBS medium. siRNAs were transfected at a concentration of 50 nM. After 6 hours, the medium containing siRNAs and Lipofectamine™ 3000 was replaced with a complete medium.

2.6. Lentiviral Overexpression of UNC5B. Lentiviral production was purchased from the GENECHEM (Shanghai, China) and infected HUVECs at PDL5 following the

Table 1: The primer sequences used in this study.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
GAPDH	TCCAAAATCAAGTGGGGCGA	AAATGAGCCCCAGCCTTCTC
IL-1α	AGATGCCTGAGATACCCAAAACC	CCAAGCACACCCAGTAGTCT
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-8	TTTTGCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC
MCP-1	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
ICAM-1	TTGGGCATAGAGACCCCGTT	GCACATTGCTCAGTTCATACACC
P53	CGCTTCGAGATGTTCCGAGA	CTTCAGGTGGCTGGAGTGAG
P21	GTCACTGTCTTGTACCCTTGTG	CGGCGTTTGGAGTGGTAGAAA
UNC5B	GTCGGACACTGCCAACTATAC	CCGCCATTCACGTAGACGAT
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
KDR	GTGATCGGAAATGACACTGGAG	CATGTTGGTCACTAACAGAAGCA

manufacturer's instructions. HUVECs were infected at a multiplicity of infection of 100 with control lentiviral vector or UNC5B-overexpressing lentiviral vector in the presence of HitansG P for 8 hours and designed as "Ctrl" or "UNC5B OE", respectively. Puromycin selection was initiated (500 ng/ml) after 3 days. qPCR and western blotting were used to detect the expression level of UNC5B in transfected cells.

2.7. Cell Proliferation Assay. Edu incorporation assay was used to detect cell growth by directly measuring DNA synthesis using iClick™ EdU Andy Fluor 555 Imaging Kit (GeneCopoeia, USA) following the manufacturer's instructions. Following cell staining, five random fields were counted and photographed under a microscope. The ImageJ software was used in the quantification of Edu-positive and DAPI-positive cells. CCK-8 test was used to monitor growth as an indirect measure for proliferation. Transfected cells were seeded in 96-well plates at a similar density and incubated for 0, 24, 48, or 72 hours. Using a SUNRISE-microplate reader, the number of viable cells after treatment with CCK-8 was evaluated by measuring the absorbance at 450 nm.

2.8. Measurement of Reactive Oxygen Species (ROS). Intracellular ROS generation was evaluated via a fluorescence microscope using dihydroethidium (DHE) fluorescent dye (MCE, China) based on the manufacturer's instructions. Briefly, HUVECs were washed with M199 and stained with DHE (10 μmol/L) at 37°C with 5% CO_2 for 30 min. After washing 3 times with M199, ethidium fluorescence was observed and photographed under an inversion fluorescence microscope.

2.9. Immunofluorescence. γ-H2AX protein expression in transfected HUVECs was detected using immunofluorescence. The cells were grown on coverslips and adequately fixed with 4% paraformaldehyde. After washing with PBS, cells were blocked in PBS with 5% goat serum and 0.5% Triton-100X at room temperature for one hour. Then, cells were incubated with primary antibody γ-H2AX (1 : 100 dilution, Cell Signaling Technology, USA) at 4°C overnight. After washing with PBS, the cells were incubated with fluorescently tagged secondary antibodies (1 : 500 dilution, Cell Signaling Technology, USA) at room temperature for 1 hour, followed by an anti-fluorescence quencher containing DAPI to seal the sections and identify nuclei. The cells were visualized and imaged using a Nikon C2 confocal microscope.

2.10. Endothelial Tube Formation and Migration Assays. The endothelial tube formation assay was performed using HUVECs. Growth factor-reduced Matrigel (Corning, USA) was spread into 96-well culture plates (50 μl/well) then allowed to rest at 37°C for 1 hour. Subsequently, transfected cells were suspended and counted using a Cellometer-Mini Automatic Cell Counter (Nexcelom Bioscience, Lawrence, MA). Cells were seeded at the density of 5000 cells/well on the Matrigel-coated plates and incubated at 37°C with 5% CO_2 for 6 hours. The network formation was photographed using an inverted light microscope. The degree of tube formation was quantified using the plugin "Angiogenesis Analyzer" from the ImageJ software.

The migration ability of HUVECs was evaluated with a transwell assay. Briefly, cells were serum-starved overnight and then seeded in the upper compartment of a 24-well transwell plate at a density of 3×10^4 cells (Corning, USA). A complete medium supplemented with 10% FBS was added to the bottom wells. HUVECs were incubated for 24 hours to allow them to migrate through the 8.0 μm polycarbonate membrane. Thereafter, cells on the lower surface of the membrane were fixed and stained with crystal violet staining solution (0.1% crystal violet, 20% methanol in PBS). The migrated cells were observed under a microscope. To quantify the signals, the bound dye was solubilized with 95% ethanol solution and the absorbance was measured at 570 nm.

2.11. Statistical Analysis. Statistical analyses were performed using the IBM SPSS software (version 22.0). Data were described as mean ± standard error of the mean (SEM) and analyzed by Student's t-test, two-way analysis of variance (ANOVA), or one-way ANOVA. A two-sided $P < 0.05$ was considered statistically significant.

3. Results

3.1. Upregulation of UNC5B in Senescent Endothelial Cells. To estimate the role of UNC5B during senescence *in vitro*,

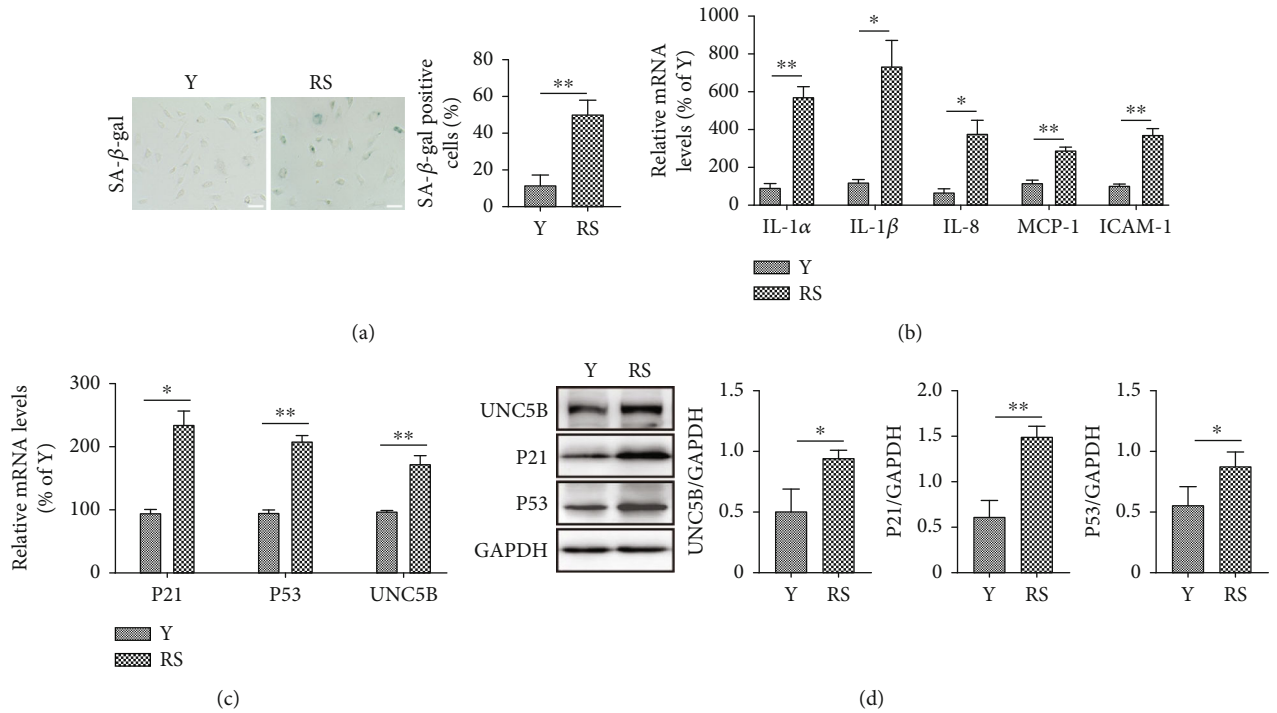


Figure 1: The expression of UNC5B is induced in senescent endothelial cells. Senescence-associated markers were used to characterize senescent endothelial cells. (a) Representative images of SA-β-gal staining assay in young (Y) and replicative senescent (RS) HUVECs. The positive cell quantification of the two groups is shown. (b) Fold changes in expression levels of the SASP mRNAs (*IL-1α*, *IL-1β*, *IL-8*, *MCP-1*, and *ICAM-1*) in senescent cells compared to young cells. (c) *P21*, *P53*, and *UNC5B* mRNA expression in young and senescent cells. (d) The protein expression of P21, P53, and UNC5B in young and senescent cells determined by western blot analysis. Scale bar, 50 μm. Quantitative data are presented as mean ± SEM of three or more independent experiments. * means $P < 0.05$; ** means $P < 0.01$.

a replicative senescent cell model was established in primary HUVECs. The SA-β-gal assay and the senescence-associated secretory phenotype genes (SASP) mRNAs were used as senescence-associated markers for identifying senescence. Consequently, unlike young cells (PDL 5-13), replicative senescent cells (PDL 25-33) demonstrated a higher proportion of blue staining (Figure 1(a)) and a significant increase in all tested SASP mRNAs including *IL-1α*, *IL-1β*, *IL-8*, *MCP-1*, and *ICAM-1* (Figure 1(b)). Meanwhile, both the mRNA and protein levels of UNC5B were significantly upregulated in senescent endothelial cells, similar to P21 and P53 (Figures 1(c) and 1(d)). Collectively, these findings indicate that UNC5B was upregulated in senescent endothelial cells.

3.2. Downregulation of UNC5B Ameliorates Endothelial Cell Senescence. To clarify the function of UNC5B in endothelial cell senescence, expression of UNC5B was downregulated in replicative senescent endothelial cells (PDL 25-33) using RNA interference. The transcriptional level of *UNC5B* was knocked down approximately 70% after siRNA transfection (Figure 2(a)). The proportion of senescent cells was lower among UNC5B-silenced cells than among the control (Figure 2(b)). The mRNA expression of the SASP genes, i.e., *IL-1α*, *IL-1β*, *IL-8*, *MCP-1*, and *ICAM-1*, was significantly attenuated by inhibition of UNC5B (Figure 2(c)). Further, the proliferation of control and UNC5B-silenced cells was assessed. As a result, UNC5B-silenced cells proliferated at a faster rate than the control cells based on the growth curve analysis (Figure 2(d)) and Edu incorporation assay (Figure 2(e)). These results reveal that the downregulation of UNC5B ameliorated the senescence-associated phenotype.

3.3. Overexpression of UNC5B Induces Premature Senescence in Young Endothelial Cells. To further explore the function of UNC5B in endothelial cell senescence, the effect of UNC5B overexpression on premature senescence was evaluated in young HUVECs (PDL 5-13). Overexpression of UNC5B was effectively achieved by lentivirus transfection (Figure 3(a)). UNC5B-overexpressing cells displayed an increase in the proportion of SA-β-gal-positive cells than control cells (Figure 3(b)). The mRNA expression of the SASP genes, i.e., *IL-1α*, *IL-1β*, *IL-8*, *MCP-1*, and *ICAM-1*, was also significantly upregulated in UNC5B-overexpressing cells compared to that of the controls (Figure 3(c)). Moreover, overexpression of UNC5B reduced total cell numbers according to growth curve analysis (Figure 3(d)) and inhibited cell proliferation potential as indicated by fewer Edu-positive cells (Figure 3(e)). These outcomes coincide with the above knockdown experiments and confirm that UNC5B is implicated in the progression of cellular senescence.

3.4. UNC5B Regulates Endothelial Cell Senescence in a ROS-Dependent Manner. Increased ROS is one of the crucial mediators of cellular senescence. Dihydroethidium staining was performed to assess whether UNC5B produced ROS.

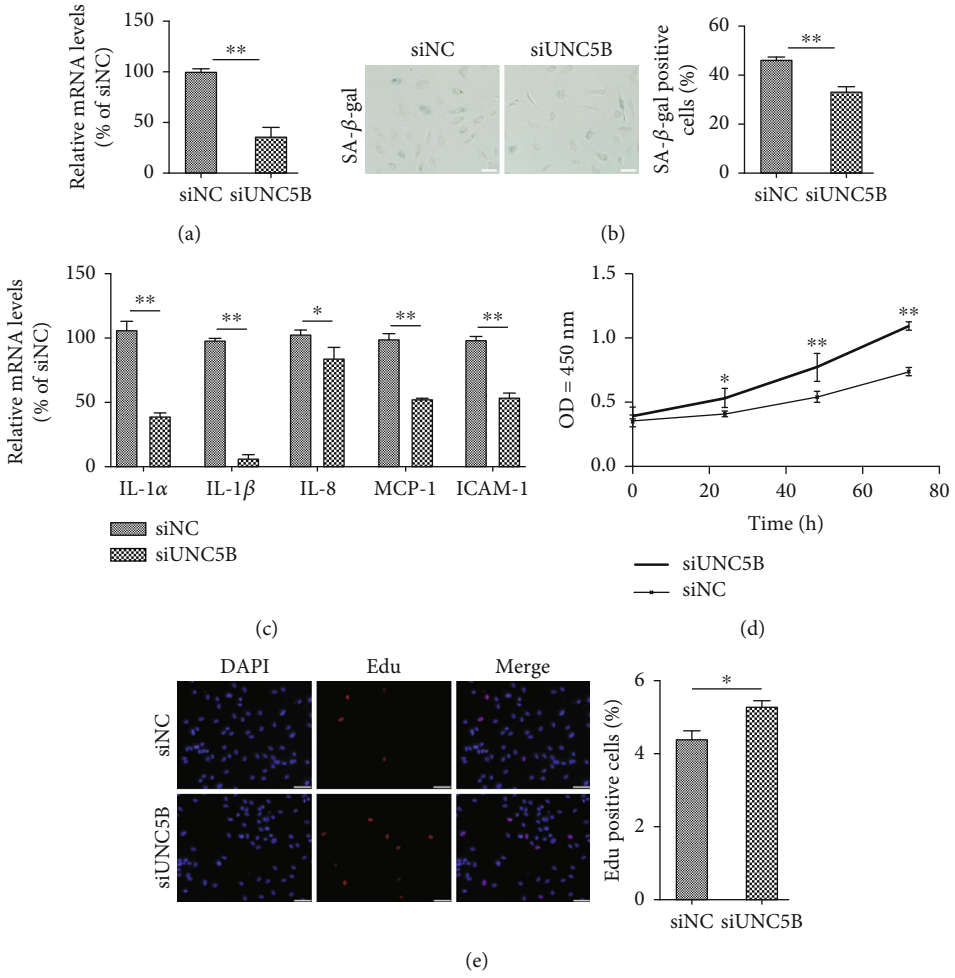


Figure 2: Downregulation of UNC5B ameliorates the senescence-associated phenotype. Replicative senescent HUVECs were transfected with negative control (NC) or siRNA UNC5B. (a) Silence efficacy confirmed by detecting the mRNA level of *UNC5B*. (b) Representative images of SA-β-gal staining assay and the percentage rate of SA-β-gal positive cells. (c) SASP evaluated by analyzing the mRNA expression levels of *IL-1α*, *IL-1β*, *IL-8*, *MCP-1*, and *ICAM-1*. (d) Growth curve analysis in transfected cells at the indicated time points. (e) Number of proliferative cells as detected by the Edu incorporation assay. Scale bar, 50 μm. Quantitative data are presented as mean ± SEM of three or more independent experiments. * means $P < 0.05$; ** means $P < 0.01$.

UNC5B-overexpressing cells demonstrated greater red fluorescence than control cells (Figure 4(a)), indicating a higher level of intracellular ROS, whereas UNC5B-silencing cells showed contrasting results (Figure 4(b)). N-acetylcysteine (NAC), a ROS scavenger, effectively rescued the ROS level induced by UNC5B overexpression (Figure 4(a)). Then, the role of UNC5B-induced intracellular ROS production was explored in the induction of senescence. Consequently, UNC5B-overexpressing cells incubated with NAC presented with fewer senescent cells than that in the absence of NAC (Figure 4(c)). Moreover, an Edu incorporation assay indicated that relatively slow proliferation was observed in control UNC5B-overexpressing cells but not in NAC presence (Figure 4(d)). Therefore, UNC5B seemingly induced senescence by increasing the intracellular ROS level.

3.5. Cell Premature Senescence Induced by UNC5B in a P53-Dependent Manner. Increased levels of intracellular ROS trigger DNA damage. Therefore, the effect of ROS accumulation induced by UNC5B was monitored on γ-H2AX nuclear foci, an indicator of DNA damage. Immunofluorescence studies revealed that approximately 32% of UNC5B-overexpressing cells were highly positive (>20 foci per nuclei) for γ-H2AX nuclear foci against 17% in control cells (Figure 5(a)). Whereas ROS inhibition by NAC prevented the formation of γ-H2AX foci in UNC5B-overexpressing cells (Figure 5(a)). Levels of γ-H2AX foci-positive cells in UNC5B-silencing cells (13%) were lower than levels in control senescing cells (27%) (Figure 5(b)). Also, UNC5B-silencing cells showed downregulated protein expression levels of P53 and P21 (Figure 5(c)). For further clarification on the relationship between the P53 pathway and senescence induced by UNC5B, this work used PFTα, a P53-specific inhibitor. The presence of PFTα inhibited the upregulation of P53 induced by UNC5B overexpression and partially suppressed the expression of P21 (Figure 5(d)). Additionally, the presence of PFTα reversed the decrease in the percentage of Edu-positive cells induced by UNC5B

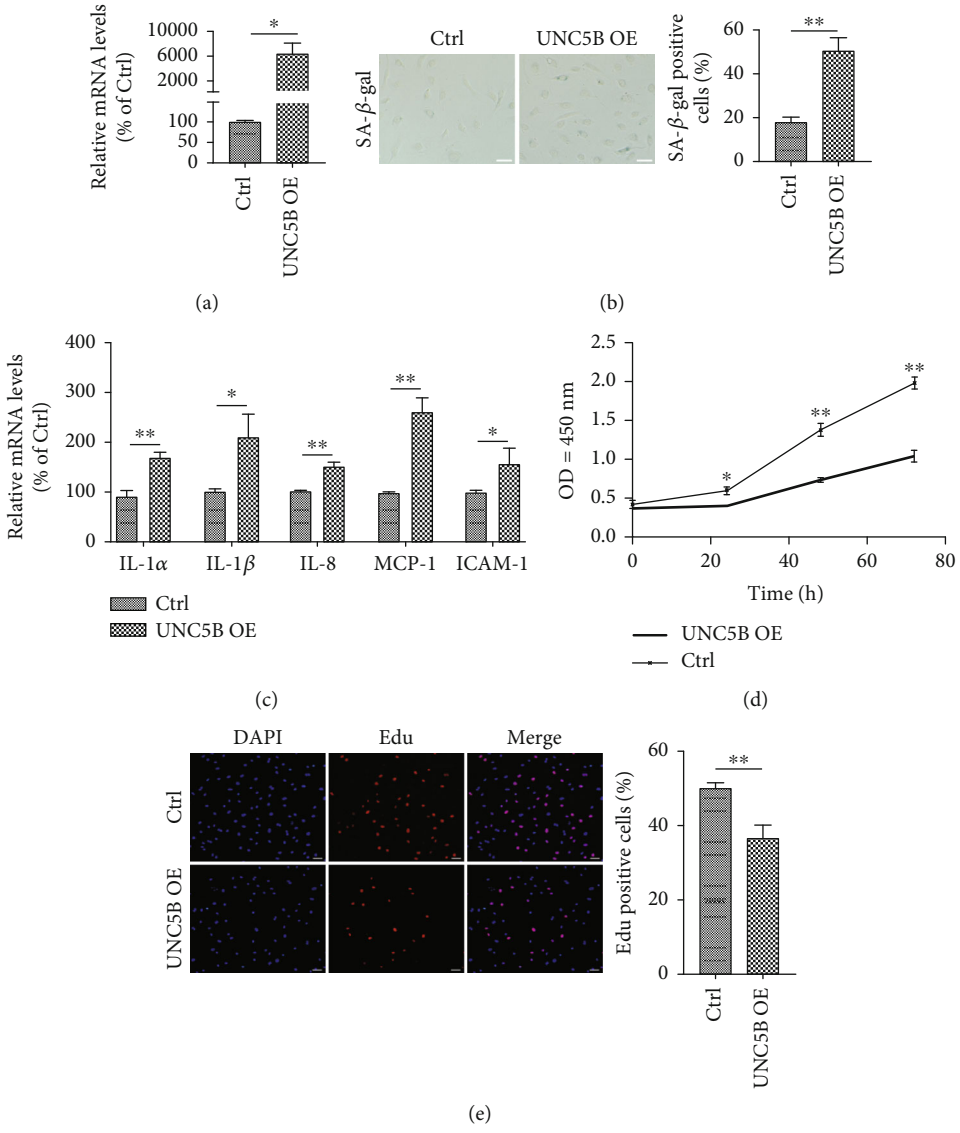


Figure 3: Overexpression of UNC5B promotes young endothelial cells undergoing premature senescence. Young HUVECs were infected with control lentiviral vectors (Ctrl) or UNC5B-overexpressing lentiviral vectors (UNC5B OE). (a) *UNC5B* mRNA levels confirmed in control cells and UNC5B-overexpressing cells. (b) The transfected cells seeded in 24-well plates for SA-β-gal staining and calculation of the percentage of positive cells. (c) Evaluation of SASP by analyzing the mRNA expression levels of *IL-1α*, *IL-1β*, *IL-8*, *MCP-1*, and *ICAM-1*. (d) Growth curve analysis in transfected cells at the indicated time points. (e) Number of proliferative cells as detected by the Edu incorporation assay. Scale bar, 50 μm. Quantitative data are presented as mean ± SEM of three or more independent experiments. * means $P < 0.05$; ** means $P < 0.01$.

overexpression (Figure 5(e)). Therefore, UNC5B could induce premature senescence through the ROS-P53 pathway in HUVECs.

3.6. UNC5B Induces Endothelial Dysfunction. Eventually, endothelial function was assessed through tube formation assay, angiogenic gene expression, and migration assay in the induction of senescence by UNC5B. For young endothelial cells, the control cells exhibited elongated and tubule-like structure, while UNC5B-overexpressing cells tube formation was hampered with an incomplete or sparse tubular network (Figure 6(a)). Inhibiting UNC5B in senescent cells increased the total length and number of junctions compared to control senescent cells (Figure 6(b)). Similarly, we found that the mRNA expression level of angiogenic genes, i.e., *VEGFA* and *KDR*, was significantly increased following UNC5B inhibition (Figure 6(c)). UNC5B-overexpressing cells exhibited decreased *VEGFA* and *KDR* mRNA levels (Figure 6(c)). The effect of UNC5B on HUVECs migration was evaluated using a transwell assay. Overexpression of UNC5B significantly impaired the migration of young cells (Figure 6(d)). Conversely, senescent cells exhibited better migratory capacity than control senescent cells following UNC5B knockdown (Figure 6(d)). These findings indicate that UNC5B disrupts endothelial function while inhibition of UNC5B enhances the vascular function.

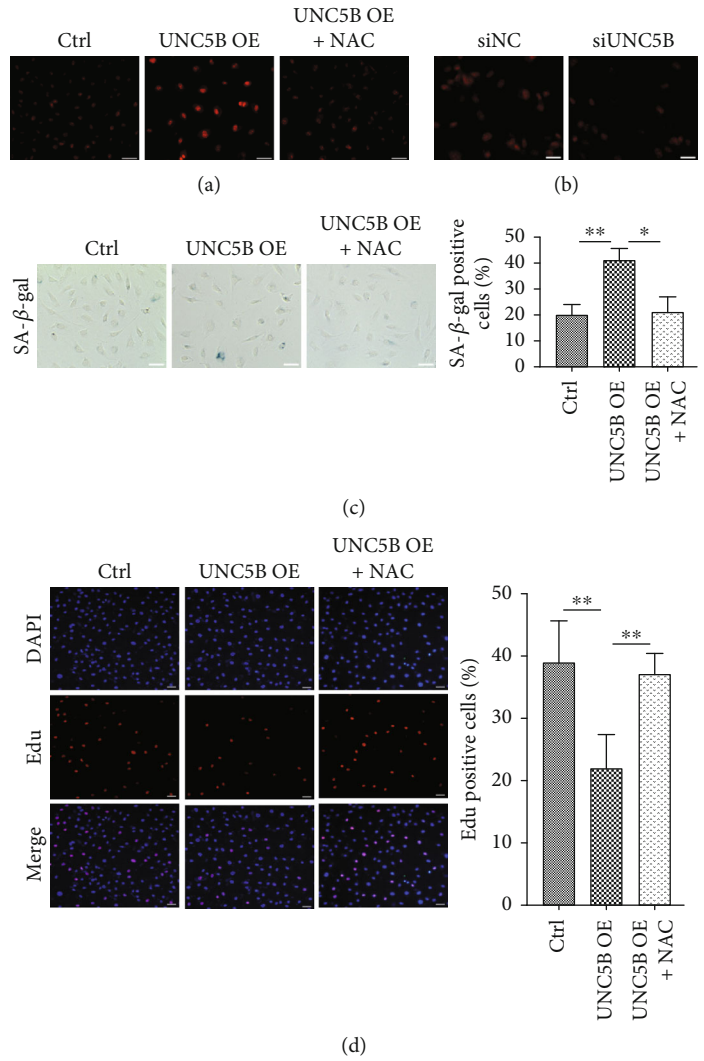


Figure 4: UNC5B induces endothelial cell senescence through ROS production. (a) Young HUVECs were prepared as Figure 3. The production of intracellular ROS was determined with DHE by microscopy. Representative images of stained cultures are shown. (b) Transfection of replicative senescent HUVECs with siRNA for three days. Determination of ROS levels in the cells. Representative images of stained cultures are shown. (c) Once infected with lentiviral vectors, cells were immediately incubated with or without NAC (5 mmol/L). The cells were then stained and analyzed for their SA-β-gal activity. (d) The proliferative potential of these cells treated with or without NAC was detected by Edu incorporation assay. Scale bar, 50 μm. Quantitative data are presented as mean ± SEM of three or more independent experiments. * means $P < 0.05$; ** means $P < 0.01$.

4. Discussion

This study demonstrated that UNC5B is a novel regulator of senescence in vascular endothelial cells. By providing morphological and functional evidence, we showed that UNC5B induced endothelial cell senescence by inhibiting cell proliferation causing endothelial dysfunction by impairing tube formation and migration. Besides, endothelial cell premature senescence induced by UNC5B was primarily ROS-dependent and activated the P53 signaling pathway.

Notably, senescent endothelial cells are found in human and rodent arterial lesions involved in atherogenesis [31]. The present data confirmed that UNC5B was upregulated in senescent cells, confirming the findings of the Tampere Vascular Study, where UNC5B was upregulated in atherosclerotic plaques [19]. Nevertheless, Tampere Vascular Study failed to provide significant evidence for the positive correlation between UNC5B and endothelial cells, possibly due to the limited number of endothelial cells in atherosclerotic plaque.

Senescent cells characteristically exhibit high SA-β-gal activity and impaired proliferation. Previous studies revealed that UNC5B overexpression inhibits proliferation in human bladder cancer cells [32, 33]. Consistently, we noted that overexpression of UNC5B in young HUVECs increased SA-β-gal activity, decreased proliferation potential, and induced the expression of cyclin-dependent kinase inhibitory proteins P53 and P21. These findings indicate that UNC5B upregulation induces premature senescence, with a possible role in cell growth blockade. Interestingly, we found UNC5B inhibition significantly prevented endothelial cell senescence, demonstrated by decreased SA-β-gal activity, increased cell

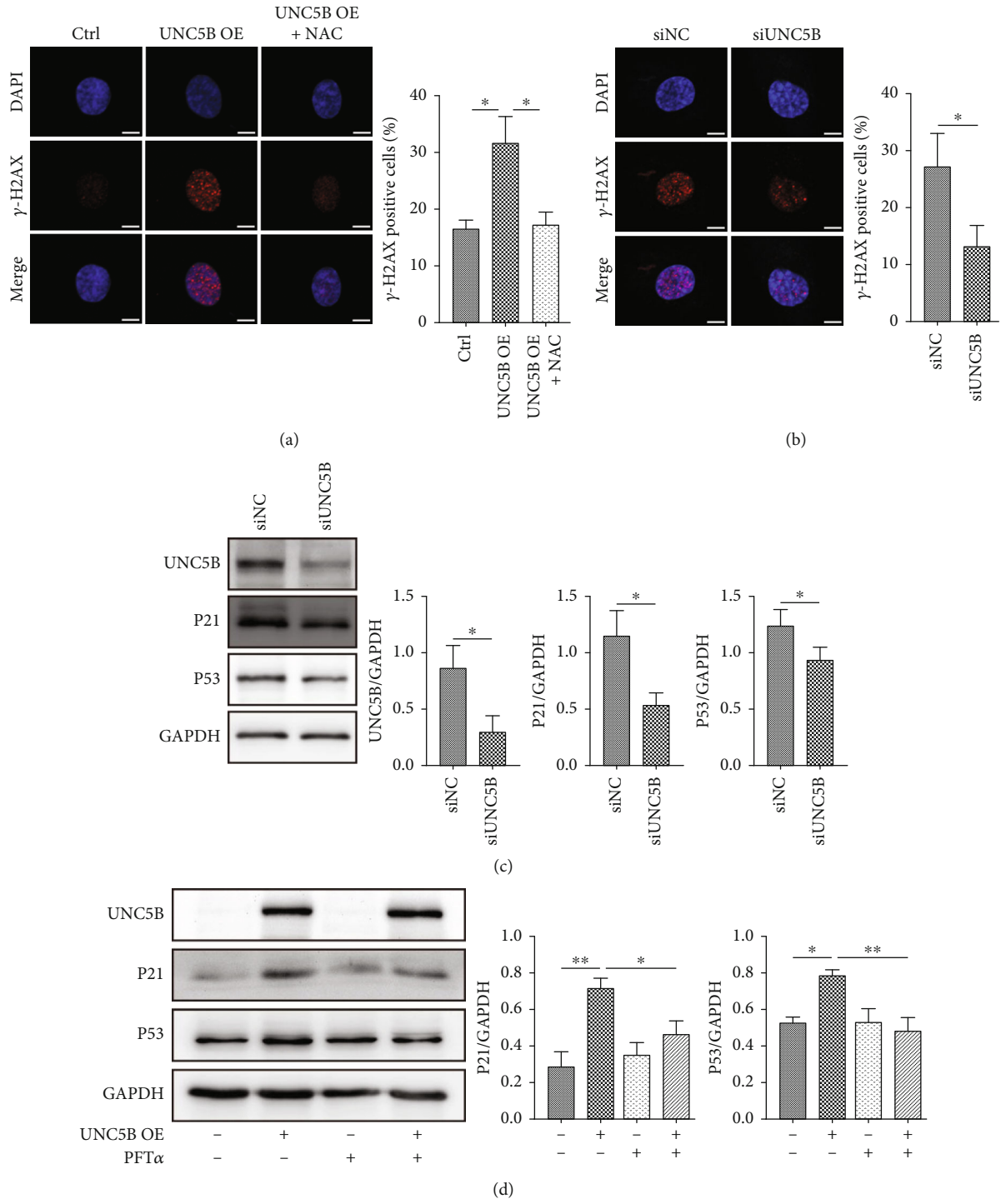
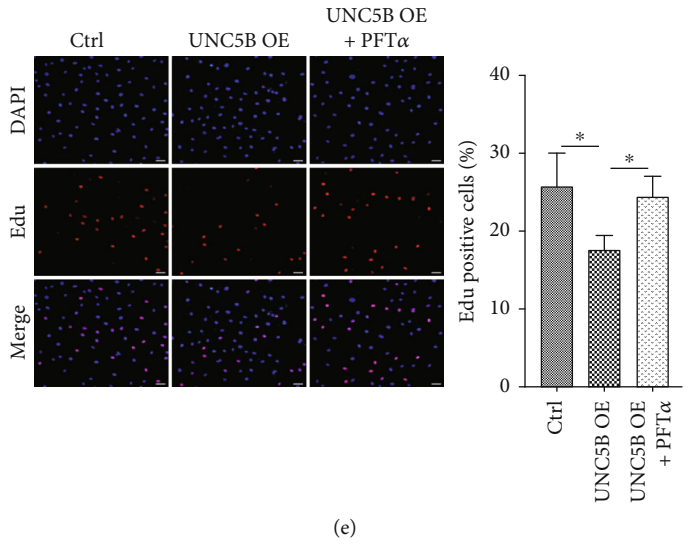


Figure 5: Continued.



(e)

Figure 5: P53 is involved in UNC5B induced endothelial cell premature senescence. (a, b) Young control and UNC5B-overexpressing HUVECs or replicative senescent HUVECs transfection with siRNA for three days were subjected to immunofluorescence of γ-H2AX foci (red). Cells with no less than 20 foci were scored as γ-H2AX-positive cells. Approximately 150 cells were analyzed per experiment. Scale bar, 10 μm. (c) Replicative senescent cells were transfected with siRNA for three days. The protein expression levels of UNC5B, P21, and P53 of the cells were analyzed by western blot. (d) After young cells infection with lentiviral vectors, PFTα or DMSO was added and maintained 5 μmol/L in both control and UNC5B-overexpressing cells throughout the experiment. The cells were then lysed for western blot analysis. (e) The proliferative potential of these cells treated with or without PFTα was detected by Edu incorporation assay. Scale bar, 50 μm. Quantitative data are presented as mean ± SEM of three or more independent experiments. * means $P < 0.05$, ** means $P < 0.01$.

proliferation, and downregulated expression of P53 and P21. In line with these data, Yang et al. reported that inhibiting the activation of the UNC5B signaling pathway in vascular endothelial cells triggered a significant increase in cell proliferation [34]. Additionally, the above study demonstrated that knockdown of UNC5B without Netrin-1 intervention did not enhance cell proliferation. This was potentially attributed to the unachieved activation of UNC5B without Netrin-1 under the reported conditions. In line with this hypothesis, UNC5B should be activated when its expression is elevated to a certain level, as observed in overexpression and in senescent cells.

Age-related increase in ROS promotes endothelial cell senescence and endothelial dysfunction [8, 35]. This increase triggers a vicious accumulation cycle of DNA damage causing further activation of ROS production, thereby, more DNA damage [35, 36]. Recent reports indicate that inhibition of UNC5B blocked the inhibitory effect of exogenous netrin-1 on oxidative stress [37], suggesting a negative effect of netrin-1/UNC5B signaling on oxidative stress. Notably, Netrin-1 was not expressed in HUVECs [38]. For the first time, we report that UNC5B regulates oxidative stress independently of Netrin-1 during senescence. Knockdown of UNC5B in senescent cells inhibited the generation of ROS, overexpression of UNC5B induced the production of ROS, while NAC treatment attenuated SA-β-gal activity and enhanced proliferation in UNC5B-overexpressing cells. As such, the mechanism of UNC5B-induced senescence was at least partly via the production of ROS. Considering the absence of exogenous netrin-1, the regulation role of UNC5B alone on oxidative stress might be incompatible with the combined effects of Netrin-1 and UNC5B. Moreover, we noted that UNC5B activated a DNA damage response (DDR) as shown in γ-H2AX foci assay and such activation was attenuated by NAC. These findings suggest a possible involvement of the DNA damage pathway in UNC5B-induced senescence.

Importantly, our results demonstrated that overexpression of UNC5B in young endothelial cells significantly activated the P53/P21 signaling pathway, while inhibition of UNC5B in senescent cells suppressed the expression of P53 and P21. UNC5B gene is a direct transcriptional target for P53 and can be activated by multiple stresses in a p53-dependent manner [23]. Previous studies showed that UNC5B mediated P53-dependent apoptosis in the absence of netrin-1 [22]. Although the mechanisms between aging and apoptosis remain unclarified, studies have speculated that apoptosis contributes to aging [39]. Besides, accumulating evidence shows that P53 plays a critical role in senescence and apoptosis. Moreover, the persistence of DDR ultimately activates P53 which triggers either senescence or apoptosis pathways via stimulation or repression of various downstream targets transcription [40–42]. Therefore, we examined whether the P53 pathway was implicated in the regulation of senescence by UNC5B. The present data revealed that the upregulated expression of P53 and P21 in UNC5B-overexpressing cells was inhibited by PFTα, an inhibitor of P53. Besides, UNC5B-overexpressing cells showed growth rescue in the presence of PFTα. Age-related senescence is triggered by P53-P21 or P16 pathways in response to DDR [43, 44]. As such, we speculated that UNC5B induces age-related endothelial cell senescence via the ROS-mediated P53 pathway.

Besides the decline in proliferation potential, senescent cells are characterized by SASP. In this study, we observed that the SASP genes were upregulated in UNC5B-

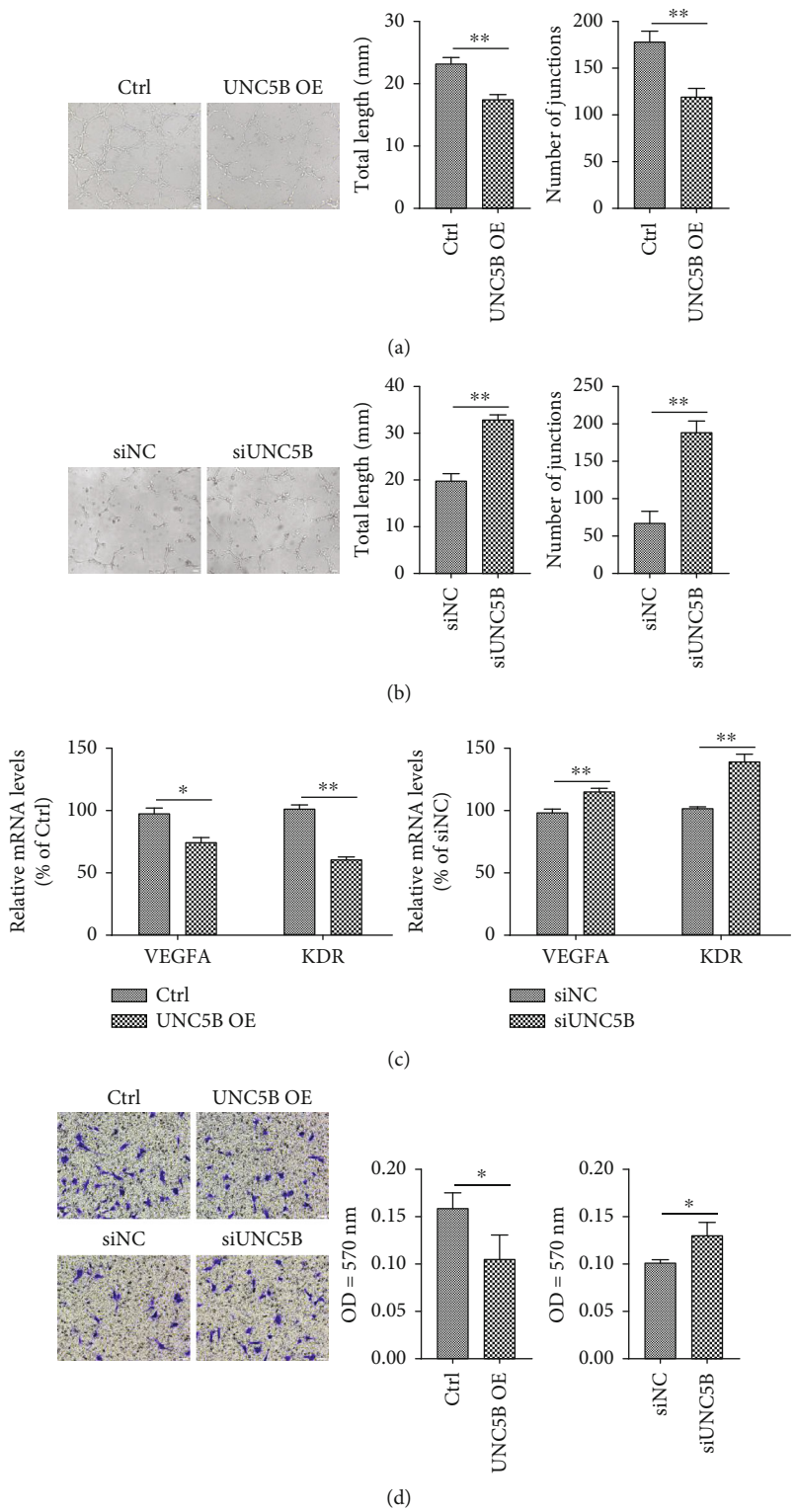


Figure 6: UNC5B impairs endothelial function. (a) In young HUVECs as prepared in Figure 3, the tube formation was observed in 96-well plates coated with Matrigel. (b) After replicative senescent cells transfection with siRNA for three days, the cells were cultured in Matrigel medium. Representative images of morphological changes of networks are shown. Lengths of the tubes and numbers of junctions were quantitated by the Image J software. (c) Angiogenic capacity evaluated by analyzing the mRNA expression levels of *VEGFA* and *KDR*. (d) Representative images of migration assay and its quantitative analysis. Scale bar, 50 μm. Quantitative data are presented as mean ± SEM of three or more independent experiments. * means $P < 0.05$, ** means $P < 0.01$.

overexpressing cells, while downregulation of UNC5B in senescent cells attenuated the expression of SASP. The SASP is primarily a persistent DDR [45]. The elevated SASP genes induced by UNC5B were further confirmed by our observation that UNC5B induced γ-H2AX formation. Therefore, the SASP activated by UNC5B might be attributed to persistent DDR signaling. The effects of UNC5B on SASP activation suggest that UNC5B might exert an effect on various cellular functions in endothelial cells.

Vascular endothelium is a vast endocrine gland of the body, secreting multiple pro-/antiangiogenic factors, cytokines, and a range of biologically active mediators [46, 47]. Previous clinical studies demonstrated that age-related arterial phenotypes are involved the progressive endothelial dysfunction [48, 49]. Impairment of angiogenic capacity is one of the major features of age-related endothelial dysfunction [50]. In our study, overexpression of UNC5B inhibited tube formation in young endothelial cells, while silencing of UNC5B in replicative senescent endothelial cells prevented senescence-associated inhibition of sprouting activity. Moreover, inhibiting UNC5B promoted senescent endothelial cell migration, which is typically associated with angiogenesis. Overexpression of UNC5B impaired the migration of young cells. Thus, we postulated that UNC5B might have antiangiogenic effects. This conclusion was further supported by results from the cell proliferation assay. Consistently, Larrivée and colleagues reported that UNC5B functioned as an antiangiogenic regulator in endothelial cells regulating developmental, postnatal, and pathological angiogenesis [15, 51]. In contrast, Navankasattusas et al. stated a proangiogenic role for UNC5B in placental arteriogenesis [52]. These conflicting findings might be attributed to the different activation states discussed earlier. Regardless, these findings indicate a pivotal role of UNC5B in angiogenesis. Our findings make contribute to clarify the molecular mechanisms of angiogenic impairment capacity in aging, which is essential for discovering novel therapeutic possibilities and prevention methods for age-related vascular diseases.

5. Conclusion

In conclusion, we found that UNC5B significantly promoted senescence observed in endothelial cells. Moreover, the ROS-P53 signaling pathway might be a mechanism of UNC5B on senescence. As such, UNC5B might be a potential therapeutic target for endothelial cell senescence and age-related vascular disorders.

Data Availability

All data used to support the findings of this study are included within the article. Raw data used to generate the figures are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

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