Oxidative Medicine and Cellular Longevity

Hormetic Effects of Exercise and Nutrition on Antioxidants and Free Radicals

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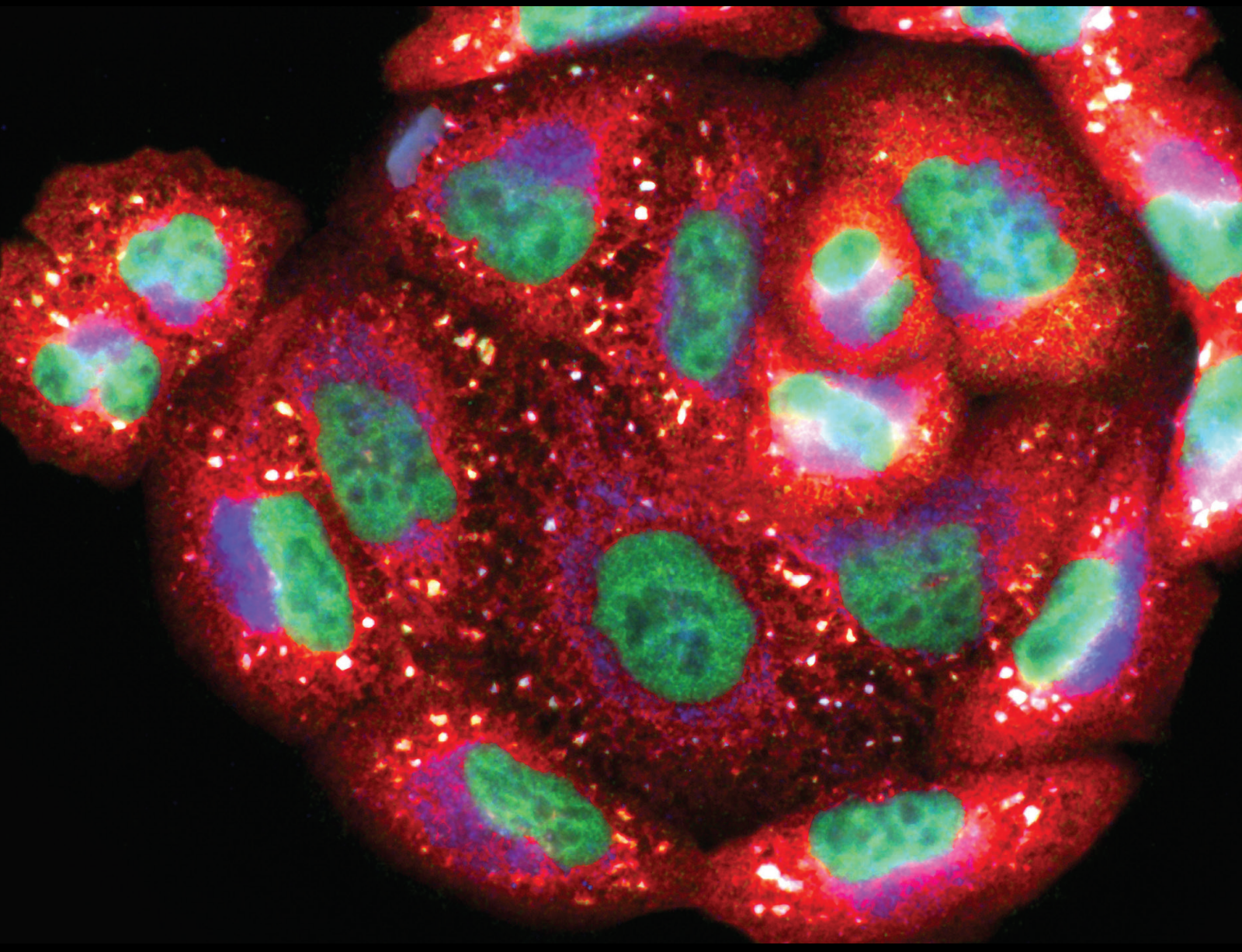

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Review Article

Hormetic Effects of Bioactive Compounds from Foods, Beverages, and Food Dressing: The Potential Role in Spinal Cord Injury

Anna Lucia Fedullo,[1] **Mario Ciccotti**,[2] **Paolo Giannotta**,[2] **Federica Alviti**,[3] **Marco Bernardi**,[4] **Anna Raguzzini**,[1] **Elisabetta Toti**,[1] **Tommaso Sciarra**,[5] **and Ilaria Peluso**[1]

[1]*Research Centre for Food and Nutrition, Council for Agricultural Research and Economics (CREA-AN), Rome, Italy*
[2]*Military Pharmaceutical Chemical Plant, Florence, Italy*
[3]*Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Board of Physical Medicine and Rehabilitation, Sapienza University of Rome, Rome, Italy*
[4]*Department of Physiology and Pharmacology "V. Erspamer", Sapienza University of Rome, Rome 00185, Italy*
[5]*Joint Veteran Center, Scientific Department, Army Medical Center, Rome, Italy*

Correspondence should be addressed to Ilaria Peluso; i.peluso@tiscali.it

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Spinal cord injury (SCI) is a damage or trauma to the spinal cord resulting in a total or partial loss of motor and sensory function. SCI is characterized by a disequilibrium between the production of reactive oxygen species and the levels of antioxidant defences, causing oxidative stress and neuroinflammation. This review is aimed at highlighting the hormetic effects of some compounds from foods, beverages, and food dressing that are able to reduce oxidative stress in patients with SCI. Although curcumin, ginseng, and green tea have been proposed for SCI management, low levels of antioxidant vitamins have been reported in individuals with SCI. Mediterranean diet includes food rich in vitamins and antioxidants. Moreover, food dressing, including spices, herbs, and extra virgin olive oil (EVOO), contains multiple components with hormetic effects. The latter involves the activation of the nuclear factor erythroid-derived 2, consequently increasing the antioxidant enzymes and decreasing inflammation. Furthermore, EVOO improves the bioavailability of carotenoids and could be a delivery system for bioactive compounds. In conclusion, Mediterranean dressing in addition to plant foods can have an important effect on redox balance in individuals with SCI.

1. Introduction

A spinal cord injury (SCI) is a condition that significantly impairs an individual's functional status, quality of life, and social independence (disability). The SCI can be divided into two main categories: the more common traumatic SCI typically caused by external physical impact [1] and nontraumatic SCI [2]. The different sites and the size of SCI can cause variable degrees of impairment from partial loss of motor or sensory function to complete paralysis below the injured spinal cord level, loss of bowel and/or bladder control, autonomic dysfunction (including in high SCI autonomic dysreflexia), and exaggerated reflex activities, as well as pain [3–8]. Based on these impairments, the interaction with the environment determines the different degrees of disability consequent to SCI. Regardless of the cause, the pathophysiology of SCI is characterized by two stages: an initial primary injury, defined as the immediate effects of an injury to the spinal cord, and a secondary progressive and self-propagating stage, characterized by multiple cascades of biochemical events in which oxidative stress is a critical component causing further tissue loss and dysfunction [3, 9–15].

The second stage is characterized by an increased formation of reactive oxygen species (ROS) and consequently by oxidative stress [16, 17]. Skeletal muscle atrophy, as well as general deconditioning, and sedentary lifestyle, commonly observed in people with SCI, can influence oxidative stress and antioxidant capacity [18, 19]. Antioxidant-based interventions have been suggested to alleviate oxidative stress and therefore to improve health in individuals with SCI [17, 20, 21].

In this context, bioactive compounds from Mediterranean diet [22, 23], as well as from beverages and food dressing [22, 24], have been proposed as hormetins, improving antioxidant defences by an hormetic mechanism mediated by the activation of the nuclear factor erythroid-derived 2 (Nrf2) antioxidant response element (ARE) pathway [22]. Many dietary components of the Mediterranean diet, such as culinary herbs and spices, as well as extra virgin olive oil (EVOO) are rich in bioactive phytochemicals [25]. Moreover, epigallocatechin-3-gallate (EGCG) from green tea, activating the Nrf2-ARE [26], is among the flavonoids suggested for treatment of SCI [23]. The aim of the present work is to review the hormetic effects of bioactive compounds from foods, beverages, and food dressing (olive oil, spices, and herbs) to reduce oxidative stress in patients with SCI.

2. Oxidative Stress in Spinal Cord Injury

Reactive nitrogen species (RNS) and ROS are produced continuously in the body, but an augmented production of ROS could exceed the capacity of the antioxidant defences (Figure 1), mediating in this way oxidative stress and subsequently oxidative damage [27, 28].

Superoxide ($O_2^{\bullet-}$), produced by the mitochondrial electron transport chain, the xanthine oxidase (XO), and the NADPH oxidase (NOX), reacts with nitric oxide ($NO^{\bullet}$), produced by the nitric oxide synthase (NOS), to form peroxynitrite ($ONOO^-$) [27, 29]. $O_2^{\bullet-}$ can be converted to hydrogen peroxide (H_2O_2) by the superoxide dismutase (SOD). The isoforms of SOD include the copper(Cu)/zinc(Zn)-SOD localized in the cytosol and in the extracellular space and the manganese(Mn)-SOD localized in the mitochondria. In this context, Zn has an essential role as part of the antioxidant defence system. Little is known about the database on the Zn status and its time-dependent changes after SCI [30–33]. A predictive model for a long-term functional outcome was obtained analyzing Zn dynamics in 38 cervically injured SCI patients [32]. Heller and colleagues [33] investigated the dynamic alterations in serum Zn concentration during the first 72 h after injury in short intervals in order to identify the relationship between the early changes of the total Zn serum level and neurological impairment and patients' outcome. They found that the median Zn concentrations in the group with neurological impairment throw down within the first 9 h after injury stronger than those in patients with vertebral fractures without neurological impairment. They concluded that the outcome is related to early Zn concentration dynamics and may be considered a helpful diagnostic indicator for these patients. In fact, the changes in serum Zn levels allow an assessment of neurological impairment risk on the first day after trauma [33]. In this regard, it was shown that Zn treatment promoted motor function recovery during the 28 days following SCI and it seems to be able to reduce ROS and enhance the antioxidant activity [34].

Despite the antioxidant effect of SOD, in the presence of iron, H_2O_2 can generate via Fenton reaction the highly reactive hydroxyl radical ($HO^{\bullet}$), initiator of the lipid peroxidation.

Both catalase (CAT) and glutathione peroxidase (GPX) catalyze the conversion of H_2O_2 into water and oxygen [35]. Among endogenous antioxidants, the main enzymes are SOD, CAT, GPX, and glutathione reductase, while glutathione (GSH) and uric acid (UA) are the major nonenzymatic antioxidants [27] (Figure 1).

GSH acts as antioxidant by scavenging ROS through GPX and by the reversible oxidation to glutathione disulphide (GSSG). The latter is reduced to GSH by the glutathione reductase. On the other hand, although XO produces $O_2^{\bullet-}$, it catalyzes the conversion of xanthine to UA which can scavenge $O_2^{\bullet-}$ and $HO^{\bullet}$ and is the major antioxidant in body fluids and preserves neuronal viability in preclinical models of SCI [36]. GPX is a selenium- (Se-) dependent enzyme and it was shown that Se nanoparticles could reverse oxidative stress-induced SCI in rats [37]. Seelig et al. [38] recently compared Se, Cu, selenoprotein P, and ceruloplasmin levels in patients with traumatic SCI versus individuals with vertebral fractures without neurological impairment and found that Cu and Se levels at admission and Se and ceruloplasmin levels after 24 h were predictors for potential remission of SCI.

Among minerals, magnesium (Mg) is suspected to have a key role in the secondary injury phase. Low Mg serum levels within the first 7 days have been described to be correlated with high probability of neurological remission [39]. In particular, Mg appears to reduce the production of ROS and lipid peroxidation [40]. Markers of the lipid product of oxidation include 4-hydroxy-2-nonenal (HNE), alkenals, alkadienals, and malondialdehyde (MDA) being the thiobarbituric acid-reactive substances (TBARS) and F2-isoprostanes (F2-IsoP) derived by the nonenzymatic oxidation of polyunsaturated fatty acids [27].

Acrolein, an aldehyde produced endogenously through lipid peroxidation implicated in SCI, is more reactive than the other HNE and induces glutathione depletion [41]. On the other hand, Bastani et al. [42] analyzed a wide panel of antioxidant and oxidative stress biomarkers to define the antioxidant status in patients with SCI. They found that the urinary F2-IsoP and some enzymes (NOX and XO) in the vastus lateralis biopsies increased in the subjects with SCI compared with the controls, whereas SOD decreased. Besides, ROS production and apoptotic signals increased 1 and 3 months after SCI, while mitochondrial complexes and the SOD-2 protein content decreased 12 months after SCI [43].

On the other hand, advanced oxidation protein products (AOPP) in plasma, cerebrospinal fluid, and the spinal cord of rats increased after SCI and triggered generation of ROS (by activating NOX), with consequent induction of the p38 mitogen-activated protein kinase (p38MAPK) and the

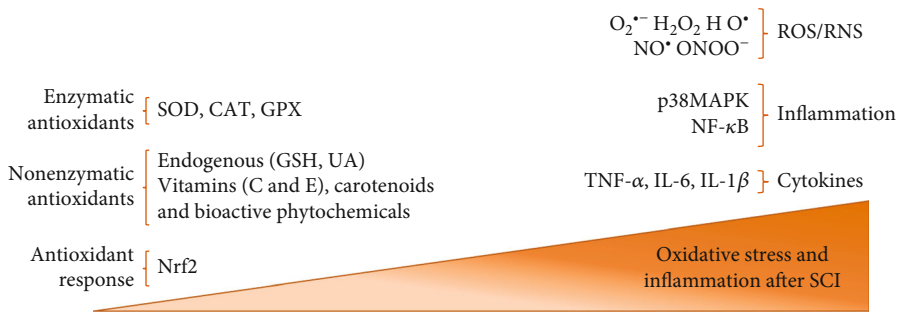

Figure 1: Representation of molecules involved in oxidative stress and inflammation after spinal cord injury (SCI). On the left are depicted as the antioxidants that are present in low concentrations, while on the right are molecules that are present at higher levels causing oxidative stress. SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; GSH: glutathione; UA: uric acid; Nrf2: nuclear factor erythroid-derived 2; $O2^{\bullet-}$: superoxide; H_2O_2: hydrogen peroxide; $HO^{\bullet}$: hydroxyl radical; $NO^{\bullet}$: nitric oxide; $ONOO^-$: peroxynitrite; p38MAPK: p38 mitogen-activated protein kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; TNF: tumor necrosis factor; IL: interleukin.

downstream regulated pathway nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and proinflammatory cytokines [44]. AOPP include protein aggregates by disulphide bridges, as well as advanced peroxidation end products and advanced glycation end products. Other markers of protein oxidation include carbonyls [45] and the derivative of tyrosine from the reaction with the hypochlorous acid (HClO), generated by the H_2O_2-dependent reaction catalyzed by the myeloperoxidase (MPO) or with $ONOO^-$ being 3-nitro-tyrosine as the main product of tyrosine oxidation [27]. Cysteine is particularly sensitive to oxidation and the reaction with $NO^{\bullet}$ produces S-nitrosylated cysteine, whereas in the presence of a proximal thiol group, ROS damage results in the formation of a disulfide bond [27]. Oxidation of cysteine residues could be an essential feature for signaling pathways, including Nrf2/ARE.

3. Dietary Antioxidants in Spinal Cord Injury

The dietary advice for individuals with SCI included Mediterranean diet [46] and an anti-inflammatory diet [47]. The latter was able to increase (after 3 months) the intake of vitamins C (ascorbic acid) and E (alpha-tocopherol) in individuals with SCI, where proinflammatory markers were negatively correlated with carotenoids [47]. Patients with this condition (from at least 2 years) showed lower serum levels of these vitamins [48] and of vitamin E and beta-carotene [49], compared with healthy controls. Vitamins (C and E) and several bioactive compounds (such as carotenoids, phenolic compounds, and glucosinolates) are exogenous antioxidants that account for the antioxidant capacity of dietary sources (Table 1).

In 1991, an innovative study, subsequently confirmed in [54, 55], proved for the first time that rats treated with vitamin E were protected against induced muscle atrophy [56]. Nevertheless, this protection seemed to be due to the downregulation of genes involved in the proteolysis of muscles, rather than by the antioxidant properties of vitamin E [57]. It has been reported that an improved bladder recovery and locomotor function in rats is associated with vitamin E-enriched diet. In fact, in order to improve sensory and autonomic dysfunctions associated with SCI, the potential use of vitamin E was suggested [58]. Moreover, vitamin E treatment markedly enhanced the hind limb locomotor function, reduced the histopathological alterations and the morphological damage in the spinal cord, and the lowered MDA level and GPX activity in SCI [59]. On the contrary, combined treatment of vitamins C and E significantly contrasted the effects of spinal cord contusion on oxidative stress, increasing SOD and GPX [60]. Recently, synergistic effects of vitamin C and taurine against SCI in rats have been investigated and the combined treatment decreased mRNA expression of interleukin- (IL-) 6, cyclooxygenase- (COX-) 2, tumor necrosis factor- (TNF-) α, and inducible NOS (iNOS) compared to the single treatments and recovered altered antioxidant markers [61]. Moreover, vitamin C treatment alone suppressed NF-κB, COX-2, and iNOS expressions in renal tissue, reduced the inflammatory responses (TNF-α and IL-1β) and oxidative stress (TBARS, protein carbonyl, and MPO), and enhanced the antioxidant status (GSH, SOD, CAT, and GPX) after SCI-induced kidney damage [62]. On the other hand, the lipid-soluble plant pigments carotenoids, having antioxidant activity, have been suggested as neuroprotective nutraceuticals [63, 64]. The carotenoid lycopene found richly in red fruits and vegetables, due to its lipophilic structure, can pass through the blood-brain barrier and reach the brain [63]. It was demonstrated that lycopene treatment in SCI rats significantly improved oxidative stress, by reversing SOD, GPX, and MDA alterations [65]. Lycopene reduced lipid peroxidation in murine models [65, 66] and NF-κB activation in a mouse model of SCI [66]. Similar inhibition of NF-κB has been reported for beta-carotene in a rat model of SCI [67]. In particular, astaxanthin, crocetin, and lycopene decreased pain [68–72]. Moreover, astaxanthin [71], crocetin [73] and crocin improved locomotor function [74].

Among flavonoids, a study conducted in mice by Borghi et al. [75] showed that quercetin could be useful to treat muscle pain conditions linked to unaccustomed exercise due to its capacity to inhibit spinal cord cytokine production, oxidative stress, and glial cell activation. Furthermore, an experimental study conducted in rats by Ocal et al. [76] suggested that quercetin can be thought as an option of treatment in

Table 1: Some common sources of antioxidants of the Mediterranean diet.

	Glucosinolates (mg/100 g)	Vitamin C (mg/100 g)	Vitamin E (mg/100 g)	Retinol equivalents (μg/100 g)	Beta-carotene (μg/100 g)	Total phenolics* (mg/100 g)
Broccoli	61.7	77	1.3	123	738	89
Brussels sprouts	236.6	81	1.0	220	1320	221
Cabbage	58.9	47	0.18	19	738	81.73
Cauliflower	43.2	59	0.15	50	114	88.63
EVOO		—	22.4	36	—	55.14
Garlic		9	—	1	6.9	87.04
Kale	100.7	110	2.24	225	1350	176.67
Onion		5	0.22	3	0	69.49
Parsley		162	1.29	943	5658	836.9
Radish	92.5	18	0	0	0	44.3
Rosemary		29	1.5	92	550	1212.3
Sage leaves		0	9.15	215	3540	1049.3
Turnip	93.0	23	2.44	0	1794	93.5

*Folin assay. Data from [50–53].

SCI. Quercetin [77] and the citrus flavonoid hesperidin [78] exerted an anti-inflammatory effect. Several studies showed that the administration of the stilbene resveratrol after SCI could provide a beneficial impact on the neurological recovery and the antioxidant activity in rats [79–83], and a recent meta-analysis of studies in rat models of SCI revealed that it increased SOD and decreased MDA levels, compared to the control group [84].

The food dressing-derived bioactive compound rosmarinic acid, identified in rosemary (987 mg/100 g) from which its name derives [25], has been suggested for SCI in a recent review [85], whereas antioxidant and/or anti-inflammatory activities in murine models of SCI have been reported for curcumin [86–88] and oleanolic acid [89].

4. The Nuclear Erythroid 2-Related Factor 2 as the Target for Spinal Cord Injury Treatment

Nrf2 is a transcription factor that regulates the antioxidant response system and inhibits oxidative stress-mediated NF-κB activation by decreasing the intracellular ROS levels [90, 91]. Normally, Nrf-2 is localized into the cytoplasm bound to the Kelch-like ECH-associating protein 1 (Keap1) that contains cysteine residues sensitive to oxidants or electrophiles [27]. Upon oxidation, Keap1 forms a disulfide bond and the conformational change results in the release of Nrf-2, allowing its translocation into the nucleus. Nrf-2 promotes the transcription of target genes containing the ARE in their promoter regions, including antioxidant enzymes and heme oxygenase 1 (HO-1). HO-1 is among the Nrf2-induced genes that inhibit NF-κB activation [90, 91]. NF-κB is normally sequestered inactive in the cytoplasm of resting cells by the inhibitor κB (IκB). The phosphorylation of two serines of IκB, by the IκB kinase (IKK), and its subsequent degradation by proteasome allow the activation of NF-κB and its translocation to the nucleus [90, 91]. After nuclear translocation, NF-κB induces the expression of proinflammatory cytokines, as well as of ROS-producing enzymes, including COX-2 and iNOS [91]. Increasing levels of TNF-α, IL-6, COX-2, and iNOS activate the Nrf2/HO-1 axis that subsequently decreases their own expressions [91]. In addition, upregulation of Nrf2 reduces the IκB-α proteasomal degradation and inhibits nuclear translocation of NF-κB [91]. NF-κB decreases the free CREB-binding protein (CBP also known as CREBBP), which is a transcriptional coactivator of Nrf2 by competing with CBP [91].

On the other hand, antioxidants with electrophilic moieties induce the Nrf2-mediated gene expression of antioxidant enzymes acting as prooxidants rather than antioxidants [26, 27, 92]. Besides, electrophilic modifications of cysteine 179 of IKK inhibit NF-κB activation and have been suggested as one of the mechanisms involved in the anti-inflammatory effects of nutraceuticals [27, 92]. Therefore, Nrf2 has a fundamental role in the hormetic effect of natural bioactive compounds (Figure 2) and its signal pathway crosstalk with the NF-κB pathway in animal models of SCI [93].

Hormetins typical of Mediterranean diet include molecules that interact with these transcription factors, such as vitamin E and many phytochemicals (terpenoids, phenolic antioxidants, allium-derived sulfur compounds, carotenoids, and resveratrol) from grapes, fruits, tomatoes, leafy green vegetables, legumes, onion, garlic, olives [22], and EVOO [94]. On the other hand, nonnutrient phytochemicals from spices often used for culinary purposes, namely, curcumin and ginger, as well as herb extracts (green tea extract, ginseng-based steroids, and ginsenosides) showed the capability to improve both oxidative stress and the inflammatory status in humans [92]. Some of these have been studied as bioactive molecules potentially useful against neurodegenerative diseases such as SCI [95].

Curcumin increased SOD levels [96] and decreased MDA [96] and proinflammatory cytokines, like TNF-α and IL-1 [97], and exerted its neuroprotective effect through the crosstalk between NF-κB and Nrf2 signaling pathways [97].

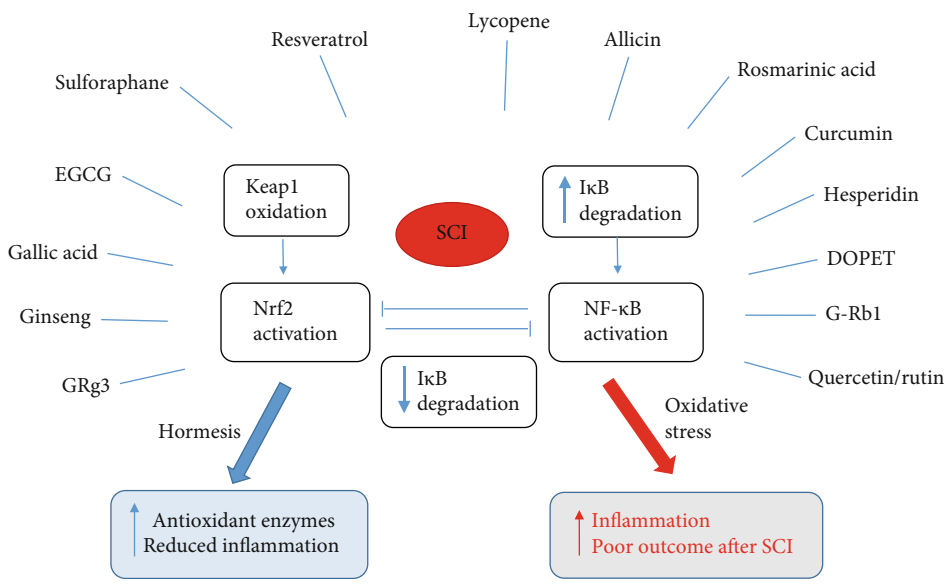

Figure 2: Bioactive compounds that act on the Nrf2/NF-κB pathway. DOPET: 3,4-dihydroxyphenylethanol; EGCG: epigallocatechin-3-gallate; GR: ginsenoside R; IκB: inhibitor κB; Keap1: Kelch-like ECH-associating protein 1; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2: nuclear factor erythroid-derived 2; SCI: spinal cord injury.

Similar effects on the NF-κB/Nrf2 pathway have been reported for sulforaphane, an isothiocyanate derived from broccoli, that is a potent naturally occurring inducer of the Keap1/Nrf2/ARE pathway and could mitigate inflammation through the inhibition of the NF-κB pathway [98]. Also, EGCG from green tea induces the Keap1/Nrf2/ARE pathway [26]. To investigate neuroprotective potential of green tea polyphenols, Zhao et al. [99] induced oxidative damage in spinal cord neurons using H_2O_2 and applied different concentrations of green tea polyphenols to the cell medium for 24 hours. Measurements of SOD activity and MDA content revealed that green tea polyphenols reduced oxidative stress [99].

Ginseng treatment significantly downregulated inflammatory markers and oxidative stress by enhancing the antioxidant status in SCI rats [100]. In particular, ginsenoside R (GR) b1 attenuates SCI-associated oxidative stress in rats by regulating the endothelial NOS/Nrf2/HO-1 signaling pathway and increased SOD, CAT, and GSH [101], whereas GR g3 show anti-inflammatory, antioxidant, and neuroprotective effects, suppressing mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and the overproduction of COX-2 and iNOS after SCI [102]. Reductions of COX-2 and NF-κB expression have been observed also with gallic acid [103], a phenolic acid contained in various plant-food sources [53]. Hesperidin, a representative flavonoid in citrus fruits, reduced proinflammatory cytokines including TNF-α and IL-1β, whereas it increased SOD, CAT, Nfr2, and HO-1 [104].

It was shown that resveratrol treatment suppressed the activation of the iNOS/p38MAPK pathway and reduced oxidative stress by enhancing enzymatic and nonenzymatic antioxidant levels such as those of GSH, SOD, and CAT in spinal cord ischemia-reperfusion injury-induced rats [105]. Furthermore, resveratrol showed a neuroprotective effect by increasing the activation of Nrf2 [106]. Preclinical studies showed that the administration of resveratrol in the acute phase or prior to experimental injury to the central nervous system could have a neuroprotective [107]. Similar results were demonstrated for quercetin. In fact, in SCI rats, quercetin has protective effects on the spinal cord by the potential mechanism of inhibiting the activation of the iNOS/p38-MAPK signaling pathway and thus regulating secondary oxidative stress [108]. Quercetin treatment reversed MDA, NO, MPO, and cytokine levels and banned the exhaustion of tissue GSH levels and SOD [109]. Also, the quercetin-3-O-rutinoside (rutin) exerts neuroprotective effects through anti-inflammatory inhibition of the p38MAPK pathway [110]. A good source of quercetin is onion [53], and among bioactive compounds from Mediterranean food dressing, there are also rosmarinic acid, allicin, and 3,4-dihydroxyphenylethanol (DOPET).

Rosmarinic acid is a water-soluble polyphenolic phytochemical that could enhance the antioxidant status and consequently decrease the oxidative stress in Wistar rats post-SCI by targeting Nrf2/HO-1 and NF-κB pathways, downregulating proinflammatory cytokines (TNF-α, IL-6, and IL-1β), and acting as neuroprotective agent [111, 112].

Garlic and onion are rich in organosulfur compounds, including allium and allicin, that induce the Nrf2 pathway [22]. Allicin, the main biologically active compound derived from garlic, seems to have neuroprotective effects in animal models, being able to increase the activities of antioxidant enzymes, including CAT, SOD, GPX, and glutathione S-transferase [113]. In addition, it was shown that allicin enhanced the motor functional recovery and increased Nrf2 nuclear expression [114], while it decreased the expression of inducible NOS but had no effects on the expression of neuronal NOS following glutamate exposure [115].

DOPET is a potent antioxidant polyphenolic compound from EVOO targeting multiple signaling pathways to reduce

SCI effects, including reduction of MPO and downregulation of proinflammatory cytokines [116]. Moreover, it has been previously reviewed that other bioactive compounds of EVOO, such as hydroxytyrosol [22, 94] and oleuropein [22], can activate the Nrf2 pathway, but specific studies are needed on SCI. Transcription of antioxidant genes mediated by Nrf2 could be also enhanced by ferulic acid (present in fruit, tomatoes, and rice), luteolin (present in carrots, peppers, and celery), phenethyl isothiocyanate (present in crucifer vegetables), and carnosic acid (abundant in rosemary) [22]. Therefore, many bioactive compounds should be tested in SCI in future studies.

5. Conclusion

SCI results, since the early stages, in an imbalance between the ROS production and antioxidant defences (Figure 1). Low levels of some micronutrients, including antioxidant vitamins and minerals involved in antioxidant enzymes' activity, have been reported in individuals with SCI. ESPEN guidelines suggested supplementation with antioxidant micronutrients for patients in the intensive care unit [117] and with neurological diseases [118] and reported in an observational study that this is practiced also in individuals with cervical SCI [119].

Dietary advice and supplements have been proposed in order to reduce oxidative stress, and in some cases, synergistic effects have been reported. Although curcumin, ginseng, and green tea have been proposed for SCI management, low levels of antioxidant vitamins have been reported in individuals with SCI. Mediterranean diet that includes food, spices, and herbs contains multiple components with antioxidant properties (Table 1), such as vitamins, phenolic compounds, and glucosinolates. The latter are known to activate Nrf2 by an electrophilic interaction with sulfhydryl-groups on Keap1, therefore in a hormetic manner. Oxidation of cysteine residue of Keap1 is involved in the EGCG induction of Nrf2. On the other hand, nonnutrient bioactive compounds from food, spices, and herbs typical of the Mediterranean diet could reduce oxidative stress by activating the Nrf2 pathway, acting as hormetins. Although many of these compounds have low bioavailability, hormetic effects typically occur at low concentration. Moreover, nanoparticle-based formulations have been suggested to improve bioavailability of flavonoids [120] and carotenoids [121] and resveratrol efficacy in SCI [122]. In particular, rats with SCI treated with resveratrol- and puerarin-loaded nanoparticles showed a decrease of GSH, SOD, and CAT antioxidant levels [122]. On the other hand, squalene from EVOO has been suggested as natural delivery system for bioactive compounds [123]. It was observed that carotenoids' absorption was higher in people that consumed salads with full-fat dressing [124]. Furthermore, EVOO is a source of vitamin E and contains many bioactive compounds [22]. From that, Mediterranean dressing in addition to plant foods can have an important effect on the redox balance in individuals with SCI. From a clinical point of view, this evidence could support the patients during both the early rehabilitation phases and the chronic management. In conclusion, the previously suggested hormetic effects of Mediterranean diet [22] that can be considered a natural multicomponent supplement [125] could be useful for the long-term management of SCI.

Abbreviations

AOPP: Advanced oxidation protein products
ARE: Antioxidant response elements
CAT: Catalase
COX: Cyclooxygenase
Cu: Copper
DOPET: 3,4-Dihydroxyphenylethanol
EGCG: Epigallocatechin-3-gallate
EVOO: Extra virgin olive oil
F2-isoP: F2 isoprostanes
GPX: Glutathione peroxidase
GR: Ginsenoside R
GSH: Glutathione
GSSG: Glutathione disulphide
H_2O_2: Hydrogen peroxide
HClO: Hypochlorous acid
HNE: 4-Hydroxy-2-nonenal
HO: Heme oxygenase
$HO^{\bullet}$: Hydroxyl radical
IL: Interleukin
IκB: Inhibitor κB
IKK: IκB kinase
iNOS: Inducible NOS
Keap1: Kelch-like ECH-associating protein 1
MAPK: Mitogen-activated protein kinase
MDA: Malondialdehyde
Mg: Magnesium
Mn: Manganese
MPO: Myeloperoxidase
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
$NO^{\bullet}$: Nitric oxide
NOS: Nitric oxide synthase
NOX: NADPH oxidase
Nrf2: Nuclear factor erythroid-derived 2
$O2^{\bullet-}$: Superoxide
$ONOO^{-}$: Peroxynitrite
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SCI: Spinal cord injury
Se: Selenium
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid-reactive substances
TNF: Tumor necrosis factor
UA: Uric acid
XO: Xanthine oxidase
Zn: Zinc.

Disclosure

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sedentary lifestyle, physical activity, and dietary habits (CISAFAL).

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

The Effect of Acute Aerobic Exercise on Redox Homeostasis and Mitochondrial Function of Rat White Adipose Tissue

Leonardo Matta,[1] Túlio S. Fonseca,[1] Caroline C. Faria,[1] Niedson Correia Lima-Junior,[1] Dahienne F. De Oliveira,[1] Leonardo Maciel,[1] Luiz F. Boa,[1] Ana Paola T. R. Pierucci,[2] Andrea C. F. Ferreira,[1,3] José H. M. Nascimento,[1] Denise P. Carvalho,[1] and Rodrigo S. Fortunato[1]

[1]*Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, 21941-590, Brazil*
[2]*Josué de Castro Institute of Nutrition, Federal University of Rio de Janeiro, Brazil*
[3]*NUMPEX, Duque de Caxias Campus, Federal University of Rio de Janeiro, Brazil*

Correspondence should be addressed to Rodrigo S. Fortunato; rsfortunato@yahoo.com.br

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Physical exercise is characterized by an increase in physical and metabolic demand in face of physical stress. It is reported that a single exercise session induces physiological responses through redox signaling to increase cellular function and energy support in diverse organs. However, little is known about the effect of a single bout of exercise on the redox homeostasis and cytoprotective gene expression of white adipose tissue (WAT). Thus, we aimed at evaluating the effects of acute aerobic exercise on WAT redox homeostasis, mitochondrial metabolism, and cytoprotective genic response. Male Wistar rats were submitted to a single moderate-high running session (treadmill) and were divided into five groups: control (CTRL, without exercise), and euthanized immediately (0 h), 30 min, 1 hour, or 2 hours after the end of the exercise session. NADPH oxidase activity was higher in 0 h and 30 min groups when compared to CTRL group. Extramitochondrial ROS production was higher in 0 h group in comparison to CTRL and 2 h groups. Mitochondrial respiration in phosphorylative state increased in 0 h group when compared to CTRL, 30 min, 1, and 2 h groups. On the other hand, mitochondrial ATP production was lower in 0 h in comparison to 30 min group, increasing in 1 and 2 h groups when compared to CTRL and 0 h groups. CAT activity was lower in all exercised groups when compared to CTRL. Regarding oxidative stress biomarkers, we observed a decrease in reduced thiol content in 0 h group compared to CTRL and 2 h groups, and higher levels of protein carbonylation in 0 and 30 min groups in comparison to the other groups. The levels returned to basal condition in 2 h group. Furthermore, aerobic exercise increased NRF2, GPX2, HMOX1, SOD1, and CAT mRNA levels. Taken together, our results suggest that one session of aerobic exercise can induce a transient prooxidative state in WAT, followed by an increase in antioxidant and cytoprotective gene expression.

1. Introduction

Physical exercise is characterized by an increase in physical and metabolic demands in face of physical stress, in a programmed, periodized, and progressive way that allows several adaptations in organic systems [1]. An acute exercise session disturbs the body's homeostasis, leading to thermal, metabolic, and oxidative stress. Several biochemical messengers are released due to these homeostatic perturbations, including Ca^{2+}, growth factors, cytokines, and reactive oxygen species (ROS). These messengers will stimulate a wide range of signaling pathways that will mediate acute and chronic responses to exercise [2]. Understanding acute responses to exercise is important for understanding how exercise conditions the body and induces long-term adaptations.

In response to exercise, an increase in skeletal muscle ROS generation is observed, mainly due to the increase of NADPH oxidase (NOX) and xanthine oxidase (XO) activities [3–5]. The transient increase in skeletal muscle ROS

induced by acute exercise activates redox-sensitive signaling pathways that are involved in many physiological responses to exercise and subsequent muscular adaptations, including the increment of antioxidant capacity [6]. Chronically, repetitive bouts of exercise improve antioxidant factor 2 (Nrf2) a transcription factor responsible to orchestrate the antioxidant defense, regulating the expression of more than 200 cytoprotective genes [7]. Interestingly, increased ROS generation and biomolecules oxidation after acute exercise are found not only in skeletal muscle but also in other organs [8, 9]. Furthermore, some pieces of evidence show that physical exercise is able to activate Nrf2/ARE (antioxidant response element) signaling and to increase antioxidant defense beyond skeletal muscle. Muthusamy et al. (2012) showed that acute exercise was able to activate Nrf2/ARE signaling and the subsequent enhancement of antioxidant defense in mouse hearts [10]. Tsou et al. (2015) evaluated if a treadmill exercise protects the nigrostriatal dopaminergic neurons by inducing Nrf2 antioxidant system in 1-methyl-4-phenylpyridine- (MPP+-) induced Parkinsonian rat model. This study showed that treadmill exercise for 4 weeks induced upregulation of Nrf2 and gamma-glutamylcysteine ligase (γGCLC) expression and also prevented the MPP +-induced downregulation of Nrf2/γGCLC/glutathione and nigrostriatal dopaminergic neurodegeneration [11]. Moreover, regular exercise has also been shown to activate Nrf2 signaling in other tissues as liver [12], kidney [13], prostate [14], and testis [15].

White adipose tissue (WAT) is a key metabolic tissue, not only important for regulating energy availability during and after exercise but also by performing an endocrine role through the secretion of adipokines, that affects the metabolism and function of different organs [16]. Physical exercise increases WAT blood flow and adrenergic stimulation that results in higher lipolysis and fatty acid oxidation rates [17, 18]. Recently, Townsend et al. (2020) showed in a genetically modified mouse model of reduced mitochondrial ROS emission (mitochondrial catalase overexpression—MCAT) that exercise-induced ROS regulates molecular responses in WAT. In wild-type mice, acute exercise induced a transient increase in *Pdk4* and phosphoenolpyruvate carboxykinase (*Pck1*) mRNA in subcutaneous inguinal WAT and epididymal WAT depots, which was not observed in MCAT animals. These results suggest that ROS is involved in WAT physiological responses to physical exercise [19]. Sakurai et al. (2009) reported that rats submitted to 9 weeks of aerobic exercise had a higher superoxide dismutase 2 expression in epididymal WAT, while the expression of the ROS-producing enzyme NOX2 and lipid peroxidation was decreased in epididymal and retroperitoneal WAT [20]. Moreover, 8 weeks of exercise training increased SOD2 and catalase protein levels and decreased lipid peroxidation in WAT of male old rats [21]. These effects of chronic physical exercise on redox homeostasis seem to be an adaptation to the acute exposure of ROS elicited by each single bout of exercise as observed in several tissues.

The studies in the literature suggest through indirect evidences that ROS could mediate the effects of acute exercise on WAT. The sources of ROS stimulated by acute exercise in WAT, as well as the modulation of redox-sensitive responses, are not completely understood. Thus, we aimed at evaluating the effect of acute aerobic exercise on WAT redox homeostasis, as well as the expression of genes related to the activation of NRF2-KEAP1-ARE pathway in male adult Wistar rats.

2. Material and Methods

2.1. Experimental Model. Adult male Wistar rats weighing 400–450 g with 18 weeks age were maintained in an animal room with controlled lighting (12-h light-dark cycle) and temperature (23–24°C) with free access to standard rat chow and drinking water. The Institutional Committee for Use of Animals in Research approved the study (Protocol n°: 132/18), and the procedures were in compliance with the International Guiding Principles for Biomedical Research Involving Animals of the Council for International Organizations of Medical Sciences (Geneva, Switzerland). The animals were divided in control group (CTRL)—without exercise and four groups (n =6/group) euthanized at different time points after the exercise session: euthanized immediately after the exercise session (0 h) and euthanized 30 minutes (30 min), 1 hour (1 h), or 2 hours after (2 h).

Prior to acute exercise, the animals of experimental groups were adapted to the treadmill for 1 week, with an average speed of 10 m/min for 10 minutes for 5 days. After the adaptation, the incremental test of maximum effort was performed with an initial speed of 6 m/min, which was increased 3 m/min every 3 minutes with fixed inclination at 10°. The test was carried out until animal's exhaustion (when the animal stayed in the steel grid despite increasing shock stimuli) adapted from Bacurau et al. (2016) [22] and according to previous studies by our group [23]. The average maximum speed reached was 34 ± 4 cm/s, and the average effort time was 30 ± 5 minutes. For the acute exercise session, the animals run between 65% and 75% of their maximum speed (obtained in the maximum effort test) for 20 minutes that corresponds to moderate-high intensity aerobic exercise. After that, the animals were euthanized by decapitation, and the retroperitoneal WAT was extracted and stored at -80°C until further analyses (maximum four weeks of storage).

2.2. Lactate Measurement Assay. Blood samples were collected from the tail vein immediately after the maximum effort test and placed in a tube with 25% sodium fluoride (NaF). The plasma was obtained by centrifugation of blood at 2,000 x g in room temperature for 15 minutes. Lactate levels were measured in plasma samples using a commercially available Bioclin kit (Quibasa, Brazil), in accordance with the manufacturers' instructions. The measurement is based on the reaction of lactic dehydrogenase (LDH) catalyzing the oxidation of L-Lactate to Pyruvate, with a consequent reduction of NAD^+ to NADH. NADH formation was measured by spectrophotometry at 340 mm in a microplate reader (Victor X4; PerkinElmer). The values were obtained through the product of the sample absorbance by the calibration factor (concentration of the standard curve × standard absorbance),

obtaining a concentration in mg/dL. The values were presented in mmol/L (lactate concentration (mg/dL) × 0.1109) [24].

2.3. NADPH Oxidase Activity. NADPH oxidase activity was quantified in the retroperitoneal WAT using the Amplex Red/Horseradish Peroxidase (HRP) Assay (Molecular Probes, Invitrogen). Retroperitoneal WAT (700 mg) was homogenized in 50 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 0.5 mM DTT, 1 mM EGTA, 5 mg/mL aprotinin, and 34.8 mg/mL PMSF. The homogenate was centrifuged at 700 x g for 10 minutes. After centrifugation, the supernatant lipid residue was discharged and the intermediate phase was collected and centrifuged at 100,000 x g for 35 minutes at 4°C. The pellet was resuspended in 0.5 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2 mM $MgCl_2$, 5 mg/mL aprotinin, and 34.8 mg/mL phenylmethanesulfonyl fluoride (PMSF) and stored at −80°C until H_2O_2 generation measurements.

The microsomal fraction was incubated in 150 mM sodium phosphate buffer (pH 7.4) containing SOD (100 U/ml; Sigma), HRP (0.5 U/mL, Roche, Indianapolis, IN), NADPH (1 mM) and Amplex Red (50 μM; Molecular Probes, Eugene, OR), and the fluorescence was immediately measured in a microplate reader (Victor X4; PerkinElmer, Norwalk, CT) at 30°C, using an excitation wavelength of 530 nm and an emission wavelength of 595 nm during 1 hour. The enzymatic activity was expressed as nanomoles of H_2O_2 per hour per milligram of protein ($nmol \cdot h^{-1} \cdot mg^{-1}$) [25]. Protein concentration was determined by the Bradford assay [26].

2.4. Mitochondria Isolation and Measurement of Mitochondrial Function. Mitochondria isolation was performed immediately after euthanasia by differential centrifugation according to the modified protocol of Maciel et al. (2020) [27]. The retroperitoneal WAT [5% tissue weight/volume (*w*/*v*)] was placed in ice-cold mitochondria-isolation buffer containing (in $mmol \cdot L^{-1}$) 250 sucrose, 10 HEPES, 1 ethylene glycol tetra acetic acid (EGTA), and pH 7.4 without bovine serum albumin (BSA). The tissue was minced carefully using scissors. Next, the minced tissue was homogenized with a tissue homogenizer (Ultra-Turrax) using two 10-sec treatments at a shaft rotation rate of 6,500 rpm. This homogenate was further homogenized using a Teflon pestle. The homogenate was centrifuged at 700 x g for 10 min at 4°C. The supernatant was collected and diluted in a cold isolation buffer containing Percoll (20%), and centrifuged at 14,000 x g for 10 min at 4° C. This procedure was repeated with isolation buffer without BSA containing Percoll (10%). The resulting pellet was resuspended in isolation buffer without BSA and Percoll and centrifuged at 10,000 x g for 5 min at 4°C. This procedure was repeated with isolation buffer without BSA and Percoll, and the pellet was resuspended in mitochondria-isolation buffer. The protein concentration of the isolated pellet was verified using a protein assay (Lowry method, Biorad, Hercules, CA, USA) by comparison to a BSA standard (Thermo Scientific, Waltham, MA, USA).

2.4.1. Mitochondrial Oxygen Consumption. Mitochondrial respiration was measured with a Clark-type electrode (Strathkelvin, Glasgow, United Kingdom) at 37°C during magnetic stirring in respiration buffer containing in $mmol \cdot L^{-1}$: 125 KCl, 10 MOPS, 2 $MgCl_2$, 5 KH_2PO_4, 0.2 EGTA with pyruvate (5 $mmol \cdot L^{-1}$), and malate (5 $mmol \cdot L^{-1}$) as substrates for complex I. The oxygen electrode was calibrated using a solubility coefficient of 217 nmol O_2/mL at 37°C. For the measurement of complex I respiration, mitochondria (corresponding to a mitochondrial protein amount of 200 μg) were added to 1 mL of incubation buffer. After 2 min of incubation, 1 $mmol \cdot L^{-1}$ ADP was added, and ADP-stimulated respiration was measured for 2 min. Mitochondria were used to either measure complex IV respiration, and maximal uncoupled oxygen uptake in the respiration chamber or the respiration buffer containing mitochondria was taken from the respiration chamber to measure ATP production and extramitochondrial ROS concentration. Complex IV respiration was stimulated by adding N,N,N,N′-tetramethyl-p-phenylenediamine (TMPD, 300 $\mu mol \cdot L^{-1}$) plus ascorbate (3 $\mu mol \cdot L^{-1}$). Maximal uncoupled oxygen uptake was measured in the presence of 30 $nmol \cdot L^{-1}$ carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) [27].

2.4.2. Mitochondrial ATP Production. After the measurement of ADP-stimulated respiration, the incubation buffer containing mitochondria was taken from the respiration chamber and immediately supplemented with the ATP Assay Mix (diluted 1 : 5) (Sigma, Aldrich). Mitochondrial ATP production after each respiration measurement was determined immediately and compared with ATP standards using a 96-well white plate and a spectrofluorometer (SpectraMax® M3, Molecular Devices, EUA) at 560 nm emission wavelength [27].

2.4.3. Extramitochondrial ROS Concentration. The Amplex Red Hydrogen Peroxide Assay Kit (Life Technologies, Carlsbad, CA, USA) was used to determine extramitochondrial ROS production. Amplex Red reacts at 1 : 1 stoichiometry with peroxides under catalysis by HRP and produces highly fluorescent resorufin. The incubation buffer containing mitochondria was removed from the respiration chamber and immediately supplemented with 50 $\mu mol \cdot L^{-1}$Amplex UltraRed and 2 U/mL HRP. The supernatant was collected after 120 min of incubation in the dark. Extramitochondrial ROS concentration was determined and compared with H_2O_2 standards using a 96-well black plate and a spectrofluorometer (SpectraMax® M3, Molecular Devices, EUA) at 540 nm emission and 580 nm extinction wavelengths [27].

2.5. Antioxidant Enzymes Activities. Retroperitoneal WAT was homogenized in 5 mM Tris-HCl buffer (pH 7.4), containing 0.9% NaCl (w/v) and 1 mM EDTA, followed by centrifugation at 750 x g for 10 minutes at 4°C. The supernatant aliquots were stored at -80° C. Total superoxide dismutase (SOD) activity was determined by reduction of cytochrome C at 550 nm [28]. Catalase (CAT) activity was assayed following the method of Aebi (1984) and was expressed as units per milligram of protein (U/mg) [29]. Glutathione peroxidase

(GPX) activity was assayed by following NADPH oxidation at 340 nm in the presence of an excess of glutathione reductase, reduced glutathione, and tert-butyl hydroperoxide as substrates and expressed as nmol of oxidized NADPH per milligram of protein (nmol/mg) [30]. Protein concentration was determined by the Bradford assay.

2.6. Biomarkers of Oxidative Damage

2.6.1. Reactive Protein Thiol Levels. Reactive protein thiol levels were measured using 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Sigma Aldrich). Thiol residues react with DTNB, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB^-), which ionizes to the NTB_2^- di-anion in water at neutral and alkaline pH. The NTB_2^- was quantified in a spectrophotometer by measuring the absorbance at 412 nm [31].

2.6.2. Lipid Peroxidation by Western Blotting. Protein samples were mixed with 2x Laemmli Sample Buffer for preparation of samples for SDS PAGE. (BioRad, Hercules, CA) and separated using a 10% Bis-Acrylamide gel at 130 V for 60-120 minutes (BioRad, Hercules, CA). Resolved proteins were then electrophoretically transferred onto nitrocellulose membranes (BioRad, Hercules, CA) at 30 V overnight. After that, to assess protein loading and transfer, membranes were incubated in 0.1% (w/v) Ponceau S in 5% acetic acid and, then, were digitally photographed by Image Quant LAS 500. The membranes were blocked for 1 h in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween) containing 3% bovine serum albumin (BSA). A 1 : 700 dilution of the primary antibody anti-4-HNE (Abcam, Cambridge, UK) was added and stirred overnight at 4°C, followed by 3 washes with 1x TBS-T. Then, the membranes were incubated for 1 h at room temperature with a 1 : 10.000 dilution of HRP-linked anti-mouse secondary antibody (Abcam, Cambridge, UK) in 3% BSA/TBS-T. The bands were visualized with Luminata™ Western HRP (Millipore, Millerica, MA) using Image Quant LAS 4000 (GE Life Science, Boston, EUA). The data were expressed as the densitometric ratio of the 4-HNE column to the total protein in each band obtained by red ponceau staining and normalized by the control group; both measures were obtained using the software Image J [32].

2.6.3. Carbonylated Proteins by 2D OxyBlot. The retroperitoneal WAT homogenate used for antioxidant analysis was denatured and derivatized with a 12% solution of sodium dodecyl sulfate (SDS) and dinitrophenylhydrazine (DNPH) according to the manufacturer's protocol (Millipore). A neutralization solution was used to terminate the derivatization reaction after 15 min. Protein separation was performed using a 12% Bis-Acrylamide gel at 120 V for 60-120 minutes (BioRad), followed by transfer to a nitrocellulose membrane at 25 V overnight. Nonspecific binding sites were blocked with 1x phosphate-buffered saline and Tween 20 (1x PBS-T) and 5% BSA for one hour. A 1 : 500 dilution of the primary antibody (Anti-Rabbit-NDP—Kit Millipore OxyBlot) was added and stirred overnight at 4°C, followed by 3 washes with 1x PBS-T. Then, the membranes were incubated with a 1 : 300 dilution of goat Anti-Rabbit IgG (conjugated with peroxidase) antibody for one hour at room temperature. The bands were visualized with Luminata™ Western HRP (Millipore, Millerica, MA) using BioRad Chemidoc and Image Lab (BioRad, Hercules, CA). The data were expressed as the densitometric ratio of the dinitrophenyl-hydrazone (DNP) bands to the total protein in each band obtained by the endogenous control tag by β-actin and normalized by control group [33].

2.7. Gene Expression by Real-Time Q-PCR. Total RNA was extracted from retroperitoneal WAT using Dynabeads™ and TissueLyser LT by disruption and homogenization through high-speed shaking of samples in 2 ml microcentrifuge. For RNA extraction, RNeasy Lipid Tissue Mini Kit (Qiagen, USA) was used following the manufacturer's instructions. RNA concentration and purity were determined by measuring the sample's absorbance at 260 and 280 nm with a spectrophotometer (Biomate 3S, Thermo Scientific), and integrity was analyzed by electrophoresis in agarose gel (1%). cDNA was synthesized from 1.2 μg of RNA in a thermocycler (Techne TC-412, UK) using High-Capacity cDNA Reverse Transcription Kit (Invitrogen, USA), according to the manufacturer's instructions. Real-time PCR was performed using EvaGreen (HOT FIREPol EvaGreen HRM mix, Solis BioDyne). The qPCR cycle was set according to the manufacturer's instructions (initial denaturation 95°C for 15 min once; followed by denaturation 95°C for 15 s plus annealing 60°-65°C for 20 s and elongation 72°C for 20 s, repeated 40 times). The β-actin gene was used as the housekeeping control gene. Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [34]. Description of primers used for real-time PCR is referred in Table 1.

2.8. Statistical Analysis. The results were expressed as mean ± standard error of the mean (SEM) and analyzed through the statistical program GraphPad Prism 7.0 (San Diego, CA, USA). The D'Agostino and Pearson test was used to verify the normality of the samples of blood lactate levels, followed by the paired t-test. Other data sets were tested for normality using the Kolmogorov-Smirnov test followed by the one-way ANOVA analysis of variance with Bonferroni multiple comparisons as posttest.

3. Results

3.1. Maximal Effort Characterization. In order to validate the maximum effort test, we measured plasma lactate concentration (Figure 1). We observed higher plasma lactate levels after the maximum effort test when compared to its levels before the test ($p < 0.0001$). This result demonstrates that all animals reached the maximum effort level, once plasma lactate was higher than 7 mmol/L, which ensures greater accuracy in the calculation of % of the maximum individual running velocity for the exercise.

3.2. Effect of Acute Aerobic Exercise on NADPH Oxidase Activity and Extramitochondrial ROS Production in Retroperitoneal WAT. Firstly, we evaluated the effect of acute aerobic exercise in the two main sources of ROS in retroperitoneal WAT: NADPH oxidase enzymes and mitochondria

Table 1: Description of primers used for real-time PCR.

GENE	Forward sequence	Reverse sequence
CAT	CAAGCTGGTTAATGCGAATGG	TTGAAAAGATCTCGGAGGCC
NFE2L2	TTTGTAGATGACCATGAGTCGC	TGTCCTGCTGTATGCTGCTT
HMOX1	ATCGTGCTCGCATGAACACT	CAGCTCCTCAAACAGCTCAATG
GCLM	CAGTGGGCACAGGTAAAACC	AATGCAGTCAAATCTGGTGGC
GPX1	AATCAGTTCGGACATCAGGAG	GAAGGTAAAGAGCGGGTGAG
GPX2	ACCGATCCCAAGCTCATCAT	TCTCAAAGTTCCAGGACACATCTG
SOD1	TGTGTCCATTGAAGATCGTGTG	CTTCCAGCATTTCCAGTCTTTG
SOD2	GGACAAACCTGAGCCCTAAG	CAAAAGACCCAAAGTCACGC
GCLC1	GGTGACGAGGTGGAGTACAT	AACATCGCCGCCATTCAGTA

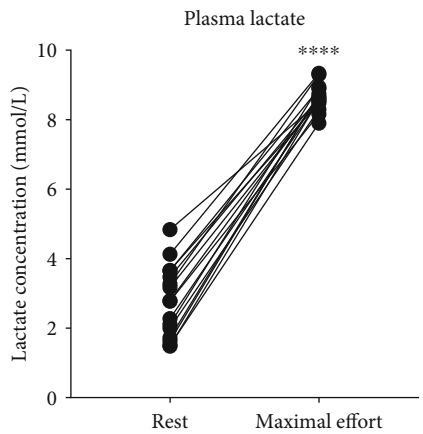

Figure 1: Lactate plasma concentration at rest and maximum postexercise in an incremental test of maximum speed. Lactate levels were measured in plasma by spectrophotometry using the BioClin ® Kit. The data were expressed as individual values ($n = 16$). $^{****}p < 0.0001$.

[35]. Regarding NOX activity, a significant increase was observed immediately and 30 minutes after exercise in comparison to CTRL group (CTRL vs. 0 h, $p < 0.05$; CTRL vs. 30 min, $p < 0.01$). However, this effect was transient, since a significant decrease in NOX activity was observed in the 2 h group in comparison to all the other exercised groups (0 h vs. 2 h: $p < 0.0001$; 30 min vs. 2 h: $p < 0.05$; and 1 h vs. 2 h: $p < 0.05$), but not to control (Figure 2(a)). In relation to the extramitochondrial ROS production, we observed a higher production of ROS immediately after the exercise session (0 h) in comparison to the CTRL group (CTRL vs. 0 h: $p < 0.05$). Interestingly, similarly to NOX activity, this effect was transient and returned to basal levels after 2 hours of the exercise session (0 h vs. 2 h: $p < 0.05$) (Figure 2(b)). These findings indicate that acute exercise stimulates a transient increase in retroperitoneal WAT ROS production mediated by NOX enzymes and mitochondria.

3.3. Acute Exercise Effects on Mitochondrial Respiration and ATP Production. Mitochondria are one of the main sources of ROS in adipose tissue [36]. Since extramitochondrial ROS production was transiently increased after exercise, we analyzed mitochondrial respiration and ATP production, in order to evaluate mitochondrial function. Our results showed that mitochondrial complex IV respiration and maximal oxygen uptake of uncoupled mitochondria were not different among groups, reflecting an equal loading of viable mitochondria in all experiments (Figure 3(a)). There were also no significant changes in mitochondrial oxygen consumption, specifically, in complex I activity, in state I (Figure 3(b)). However, we observed that in state II of complex I, the 0 h group had a higher consumption of O_2 in relation to all the other groups (0 h vs. CTRL, $p < 0.01$; 0 h vs. 30 min, $p < 0.004$; vs. 1 h, $p < 0.009$ and vs. 2 h, $p < 0.001$) (Figure 3(c)). In addition, when analyzing state III of complex I, we found that immediately after exercise (0 h) O_2 consumption was significantly higher compared to the other groups (0 h vs. CTRL, $p < 0.0001$; vs. 30 min, $p < 0.001$; vs. 1 h and 2 h: $p < 0.0001$) (Figure 3(d)). Interestingly, we found that despite the high oxygen mitochondrial consumption in the phosphorylative state of complex I in 0 h group, it was not accompanied by an increase in ATP production. Instead, ATP production had a tendency to reduce immediately after exercise when compared to control group (CTRL vs. 0 h, $p = 0.0518$), thus suggesting that the oxygen consumed by mitochondria is deviated to ROS production instead of ATP synthesis. In 30 min group, it was demonstrated that ATP production increased significantly compared to 0 h (0 h vs. 30 min: $p < 0.05$). ATP production of 1 h group was higher than CTRL (CTRL vs. 1 h, $p < 0.05$) and 0 h groups (0 h vs. 1 h, $p < 0.0001$), as well as 2 h group (CTRL vs. 2 h, $p < 0.05$: CTRL vs. 0 h, $p < 0.0001$). No differences were found between 1 h and 2 h groups (Figure 3(e)). These results suggest a negative relationship between ROS and ATP production in mitochondria.

3.4. Antioxidant Enzymes Activities. ROS availability in a given tissue depends on their production and detoxification rates. Since our results demonstrate an increase in ROS production after exercise, we decided to evaluate the activity of the antioxidant enzymes CAT, SOD, and GPX. CAT activity was lower in all exercised groups in comparison to control (CTRL vs. 0 h, $p < 0.001$; vs. 30 min, $p < 0.01$; vs. 1 h, $p < 0.001$; vs. 2 h, $p < 0.0001$) (Figure 4(a)). However, no differences were observed among groups for GPX (Figure 4(b)) and SOD (Figure 4(c)) activities.

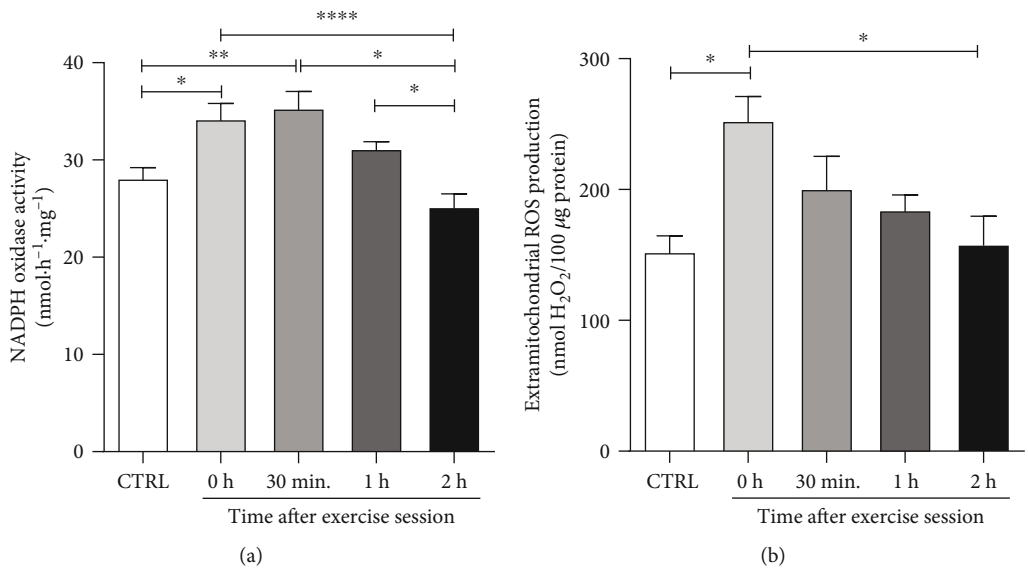

Figure 2: The effects of an acute exercise on the main sources of ROS of retroperitoneal WAT in rats. The animals were submitted to aerobic training on a treadmill for 20 minutes at an intensity of 75% of maximum speed and euthanized at different time points. (a) NADPH oxidase activity was measured by spectrophotometry in microsomal fraction ($n = 7$/group). (b) Generation of mitochondrial H_2O_2 measured by spectrophotometry ($n = 4$/group). CTRL: control; 0 h; euthanasia immediately after exercise; 30 min: euthanasia 30 minutes after exercise; 1 h: euthanasia 1 hour after exercise; 2 h: euthanasia 2 hours after exercise. The data were expressed as the mean ± standard error of the mean ($n = 7$/group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

3.5. Biomarkers of Oxidative Damage. Since we observed an increase in NOX and mitochondrial ROS production accompanied by decreased CAT activity immediately after exercise session, we decided to measure two biomarkers of oxidative damage to indirectly evaluate ROS availability and the direct impact of ROS in biological macromolecules [37]. Based on this, we analyzed three specific markers of biomolecule oxidation: reactive protein thiol, protein carbonyl, and lipid peroxidation levels. The levels of reactive protein thiols were lower in 0 h group when compared to control (CTRL vs. 0 h, $p < 0.01$) and 2 h group (0 h vs. 2 h, $p < 0.05$). No differences were observed among CTRL, 30 min, 1 h, and 2 h groups (Figure 5(a)). These results suggest that exercise elicited a prooxidative environment in WAT immediately after the session (observed by the oxidation of thiol groups) that was followed by a return to baseline levels.

Lipid peroxidation levels were higher in 0 h (CTRL vs. 0 h, $p < 0.05$), 0.5 h group (CTRL vs. 30 min, $p < 0.05$), and 1 h group (CTRL vs. 1 h, $p < 0.01$) when compared to control (Figure 5(b)). Besides that, protein carbonyl levels were higher in 0 h (CTRL vs. 0 h, $p < 0.01$) and 0.5 h. groups (CTRL vs. 30 min, $p < 0.01$) in comparison to control. In 1 h and 2 h groups, protein carbonylation levels have decreased in relation to 0 h (0 h vs. 1 h, $p < 0.0001$; 0 h vs. 2 h, $p < 0.0001$) and 30 min (30 min vs. 1 h, $p < 0.0001$; 30 min vs. 2 h, $p < 0.0001$) (Figure 5(c)). These results are in line with reactive protein thiol levels, suggesting a transient pro-oxidative state after exercise session.

3.6. Effects on Antioxidants Gene Expression. Physical exercise can activate redox-sensitive intracellular signaling pathways through ROS-related mechanisms in several tissues, leading to physiological modifications through both genomic and non-genomic mechanisms [38]. Since we observed that our exercise protocol resulted in increased ROS availability in retroperitoneal WAT, we analyzed if antioxidant and cytoprotective genes related to the activation of NRF2-KEAP1 pathway would be modulated (Figure 6).

One hour after exercise (1 h), a significant increase in NRF2 mRNA levels was observed in relation to the CTRL ($p < 0.05$), 0 h ($p < 0.001$), and 2 h ($p < 0.05$) groups (Figure 6(a)). GPX2 mRNA levels were higher in 30 min group in relation to 1 h ($p < 0.05$) and 2 h ($p < 0.01$) groups (Figure 6(c)). Glutamate-cysteine ligase modifying subunits (GCLM) mRNA levels were lower in 1 h group when compared to CTRL ($p < 0.05$) and 0 h ($p < 0.05$) groups (Figure 6(d)). Heme oxygenase1 1 (HMOX1) mRNA levels were higher in 1 h group in relation to the CTRL ($p < 0.05$), 0 h ($p < 0.001$), 30 min ($p < 0.05$), and 2 h ($p < 0.01$) groups (Figure 6(f)). SOD1 gene expression was higher in 2 h group in comparison to CTRL ($p < 0.01$), 0 h ($p < 0.01$), 30 min ($p < 001$), and 1 h ($p < 0.01$) groups (Figure 6(g)). It was also observed a significant increase in CAT expression in 0 h in relation to CTRL ($p < 0.05$), 30 min ($p < 0.05$), 1 h ($p < 0.01$), and 2 h ($p < 0.01$) groups (Figure 6(i)), thus suggesting that the reduction of catalase activity after exercise (Figure 5(a)) was due to posttranslational mechanisms. There were no significant changes of SOD2, Catalytic Subunit 1 Glutamate-Cysteine Ligase (GCLC1) and GPX1 mRNA levels in any of the postexercise periods evaluated. These results show that one acute aerobic exercise session was able to modulate the mRNA levels of some antioxidant and cytoprotective genes.

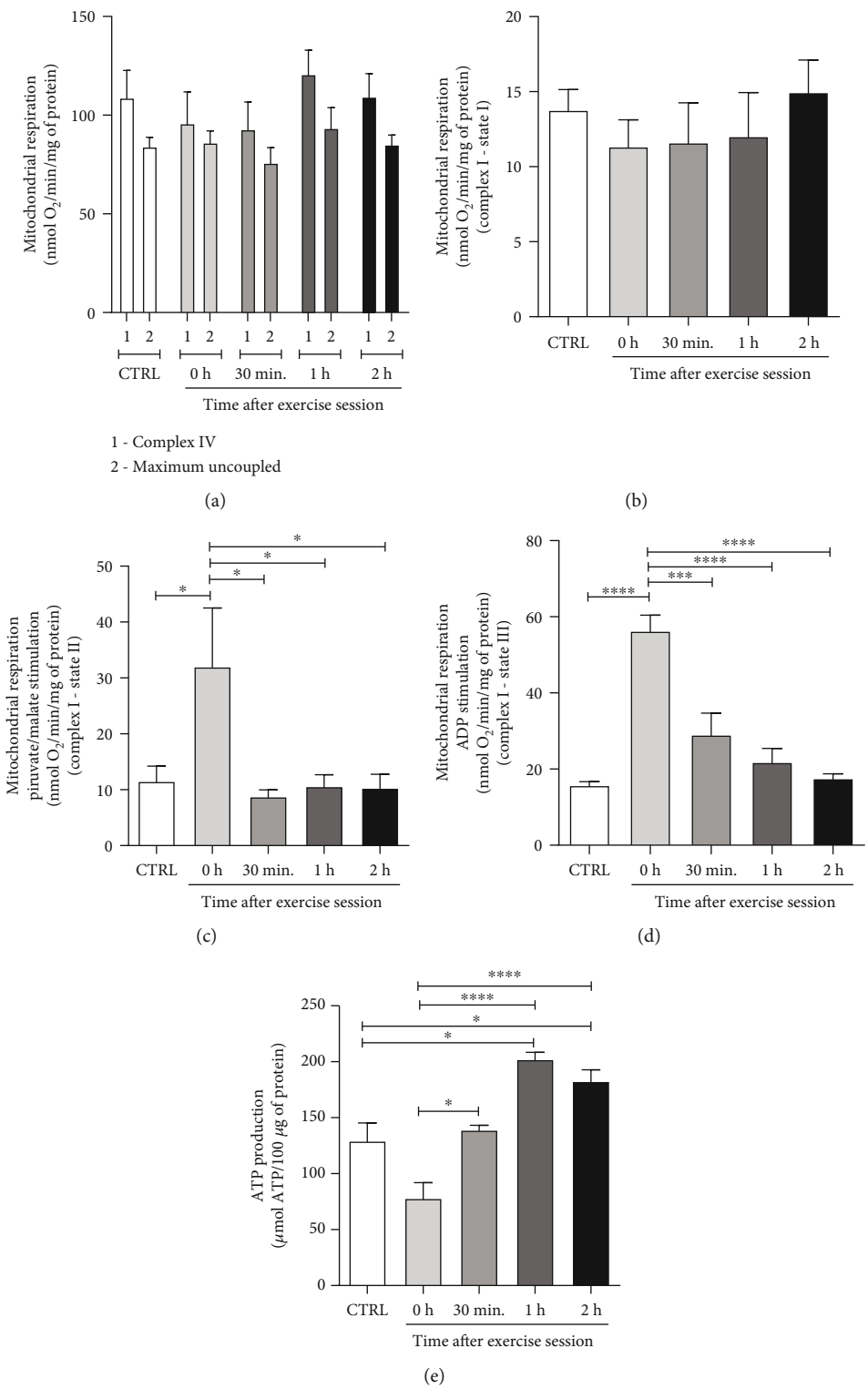

Figure 3: Effect of an acute exercise session on mitochondrial respiration and ATP production levels on retroperitoneal WAT in rats. (a) Mitochondrial oxygen consumption at baseline and maximum levels, FCCP was added at 30 μmol to assess the maximum consumption of O_2. (b) Mitochondrial respiration at rest, free of complex I activation substrate. (c) Mitochondrial respiration in state II of complex I, stimulated by the addition of pyruvate (5 mmol·L^{-1}) and malate (5 mmol·L^{-1}). (d) Mitochondrial respiration in state III of complex I (oxidative phosphorylation) stimulated by the addition of ADP (1 mmol·L^{-1}) for 2 minutes. (e) ATP production by spectrophotometry. CTRL: control; 0 h; euthanasia immediately after exercise; 30 min: euthanasia 30 minutes after exercise; 1 h: euthanasia 1 hour after exercise; 2 h: euthanasia 2 hours after exercise. The data were expressed as the mean ± standard error of the mean ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

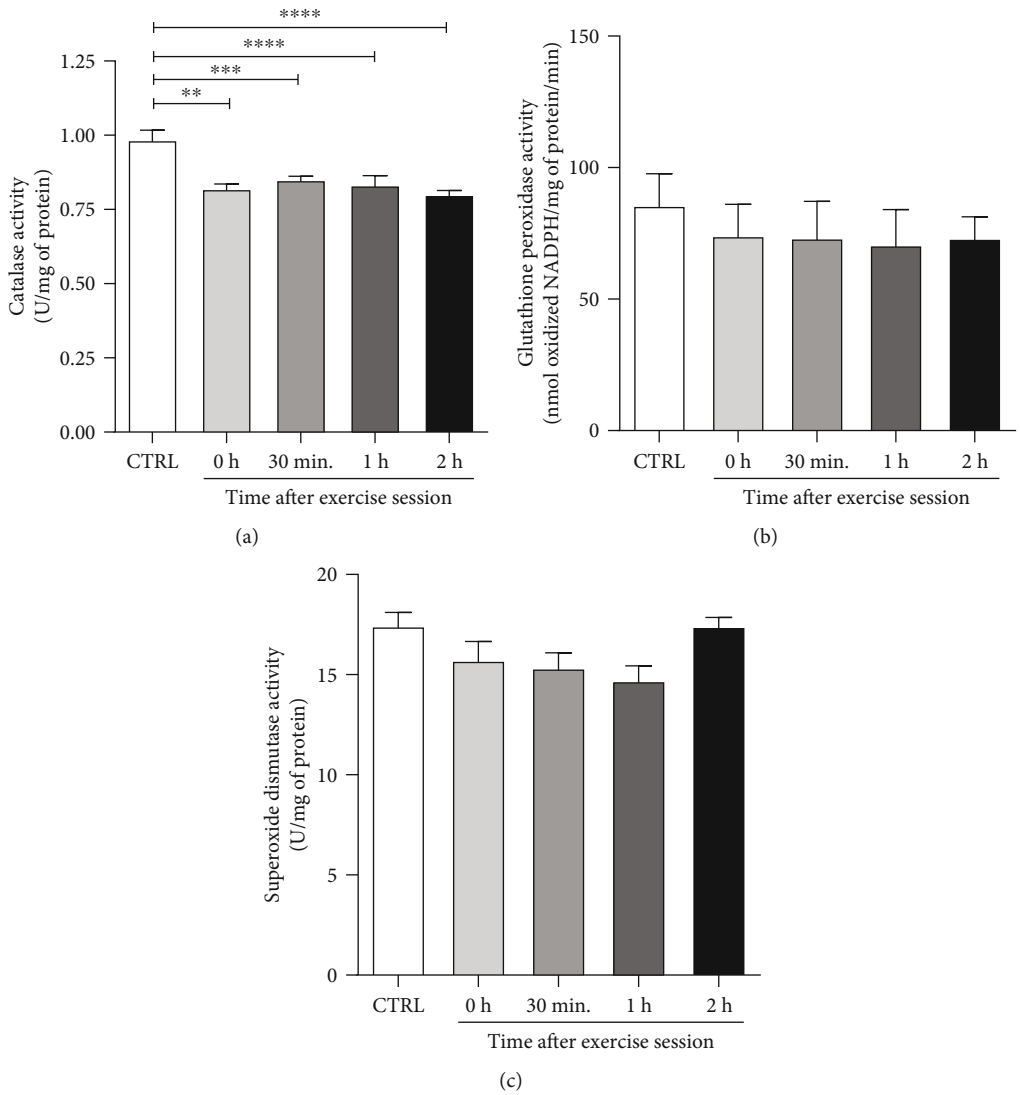

Figure 4: Effect of an acute exercise session on the antioxidant enzymatic activity of retroperitoneal WAT in rats. (a) Catalase, (b) GPX, and (c) SOD activities were measured in retroperitoneal WAT homogenates by spectrophotometry. CTRL: control; 0 h; euthanasia immediately after exercise; 30 min: euthanasia 30 minutes after exercise; 1 h: euthanasia 1 hour after exercise; 2 h: euthanasia 2 hours after exercise. The data were expressed as the mean ± standard error of the mean ($n = 7$). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

4. Discussion

Several studies have shown that chronic exposure to exercise results in enhanced cytoprotection and antioxidant defenses in different tissues, such as skeletal muscle [10], heart [39], brain [40], and others [41]. This physiological adaptation is related to the induction of low levels of ROS in each single bout of exercise [38]. In WAT, there are several pieces of evidence showing that chronic exposure to aerobic exercise is linked to lipid storage reduction, fatty acid mobilization [42], improved mitochondrial function [43], decreased expression of inflammatory adipokines [20], and consequently modified WAT metabolism [44], and phenotype [45]. Chronic exercise seems to elicit redox adaptations in WAT, such as decreased ROS production and increased antioxidant defense [46]. Our study revealed that acute moderate-high intensity endurance exercise promoted a transient prooxidative state in WAT, which resulted in transient oxidation of biomolecules, and increased antioxidant/-cytoprotective gene expression.

In the present study, we evaluated two important sources of ROS in WAT, NOX enzymes and mitochondria [47, 48]. NOXs are enzymes whose only function is the production of superoxide or H_2O_2 [49]. They are transmembrane enzymes and belong to the NOX family, including NOXs from 1 to 5, and DUOXs 1 and 2, which show different tissue distribution and expression levels [50]. NOX2 and NOX4 seem to be the most expressed NOX isoforms in WAT [51], but we could not find any evidence in the literature about the effect of acute exercise on their activities. In the present work, we observed a transient increase in NOX-derived ROS production, which was higher immediately after the exercise session and also 30 minutes later, then returning to baseline levels. NOX enzymes can be activated by several

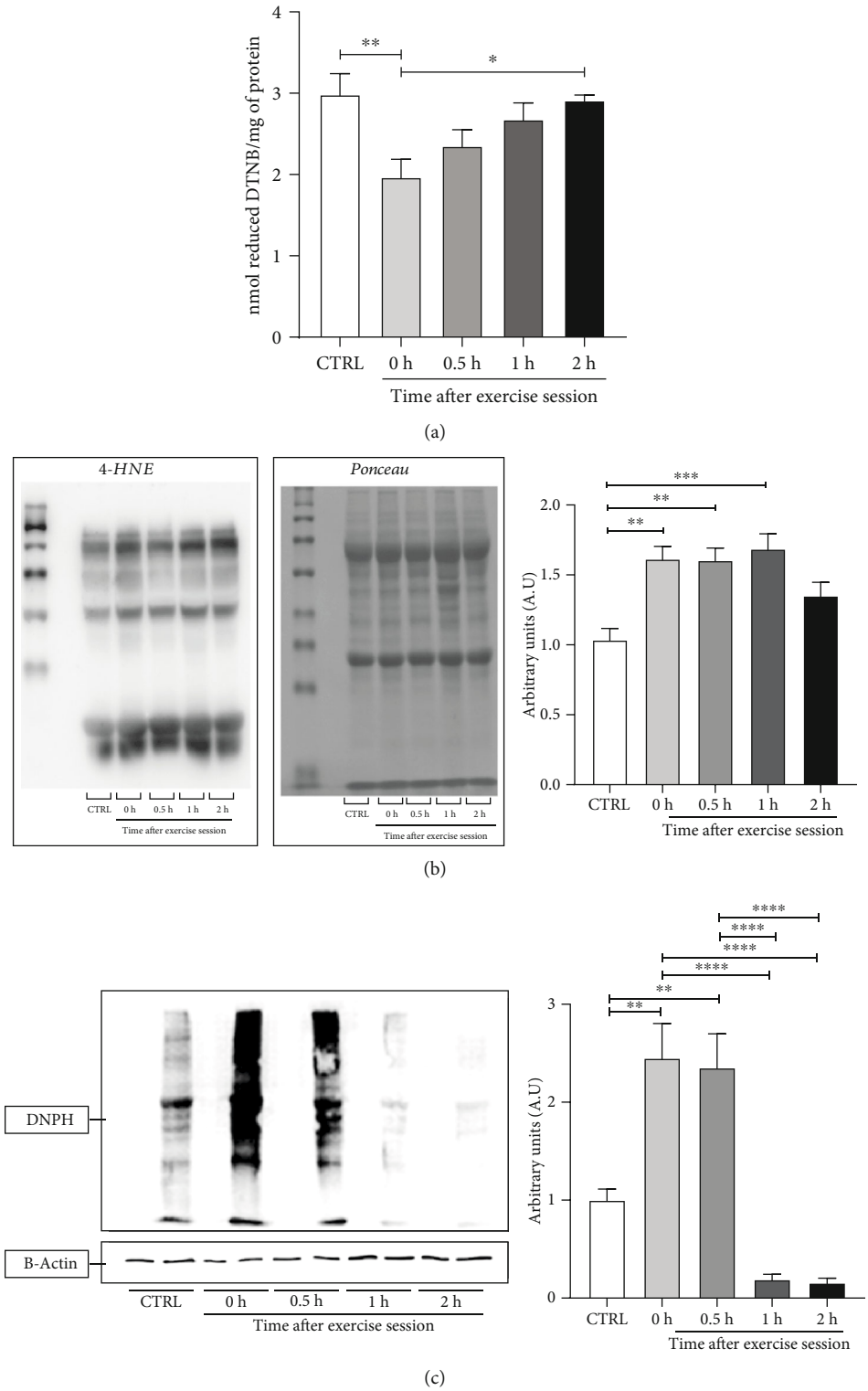

Figure 5: Effect of an acute exercise session on biomarkers of oxidative damage in the retroperitoneal WAT of rats. (a) Levels of reduced thiol content. Total sulfhydryl groups were measured in homogenates of retroperitoneal WAT by spectrophotometry. DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid) ($n = 7$/group). (b) Levels of lipid peroxidation, representative result of a membrane incubated with anti-4-HNE and Ponceau Rouge. Data were normalized to Ponceau Rouge and expressed as relative to control. 4-HNE: 4-hydroxynonenal ($n = 6$/group). (c) Levels of carbonylated proteins, representative result of a membrane incubated with anti-DNPH and anti-β-actin using the OxyBlot® Kit. Data were normalized to β-actin and expressed as relative to control. DNPH: 2,4-dinitrophenylhydrazine. CTRL: control; 0 h; euthanasia immediately after exercise; 30 min: euthanasia 30 minutes after exercise; 1 h: euthanasia 1 hour after exercise; 2 h: euthanasia 2 hours after exercise. The data were expressed as the mean ± standard error of the mean ($n = 5$/group). $^{*}p < 0.05$; $^{**}p < 0{,}005$, $^{***}p < 0.01$; $^{****}p < 0.0001$.

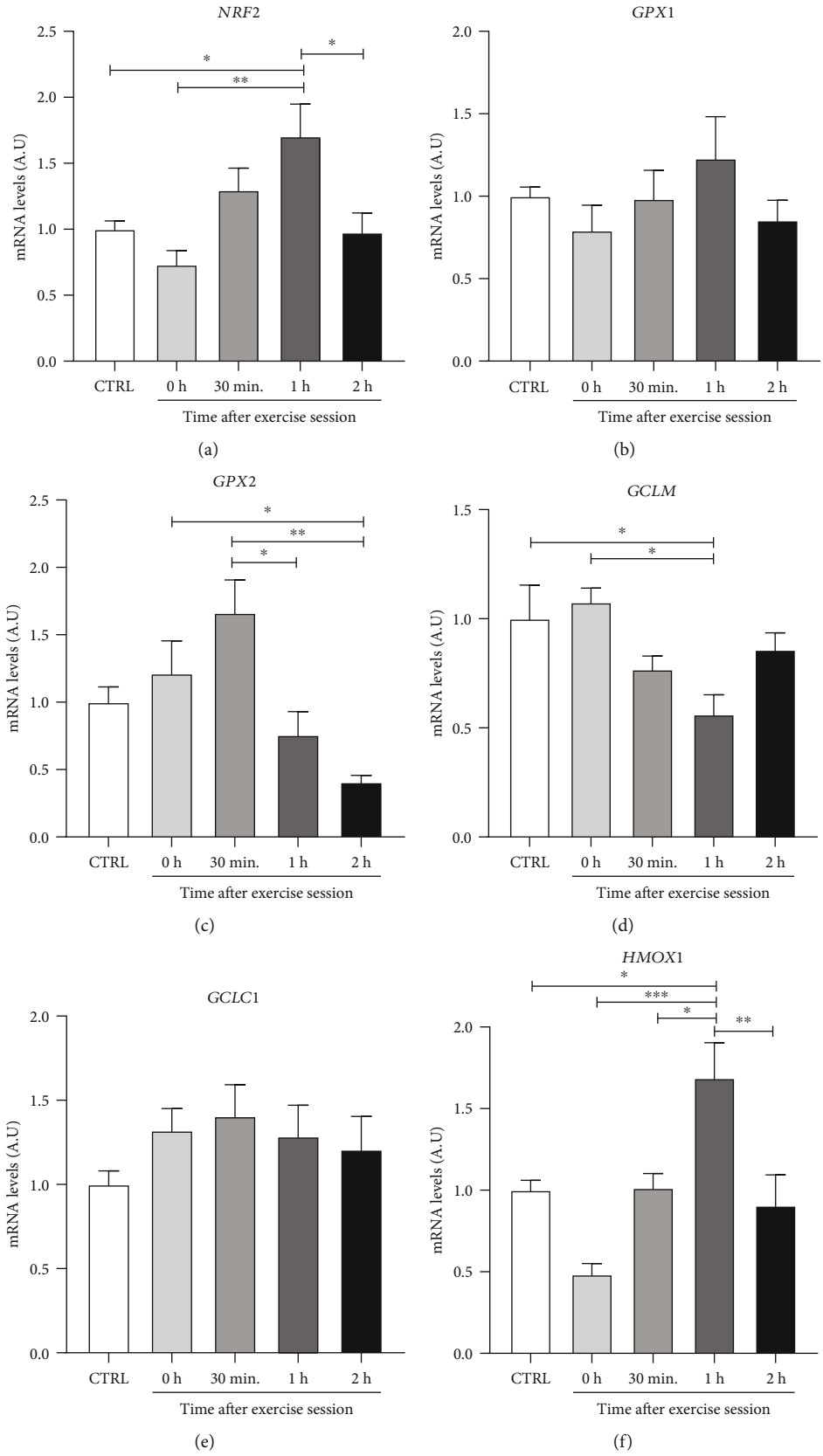

Figure 6: Continued.

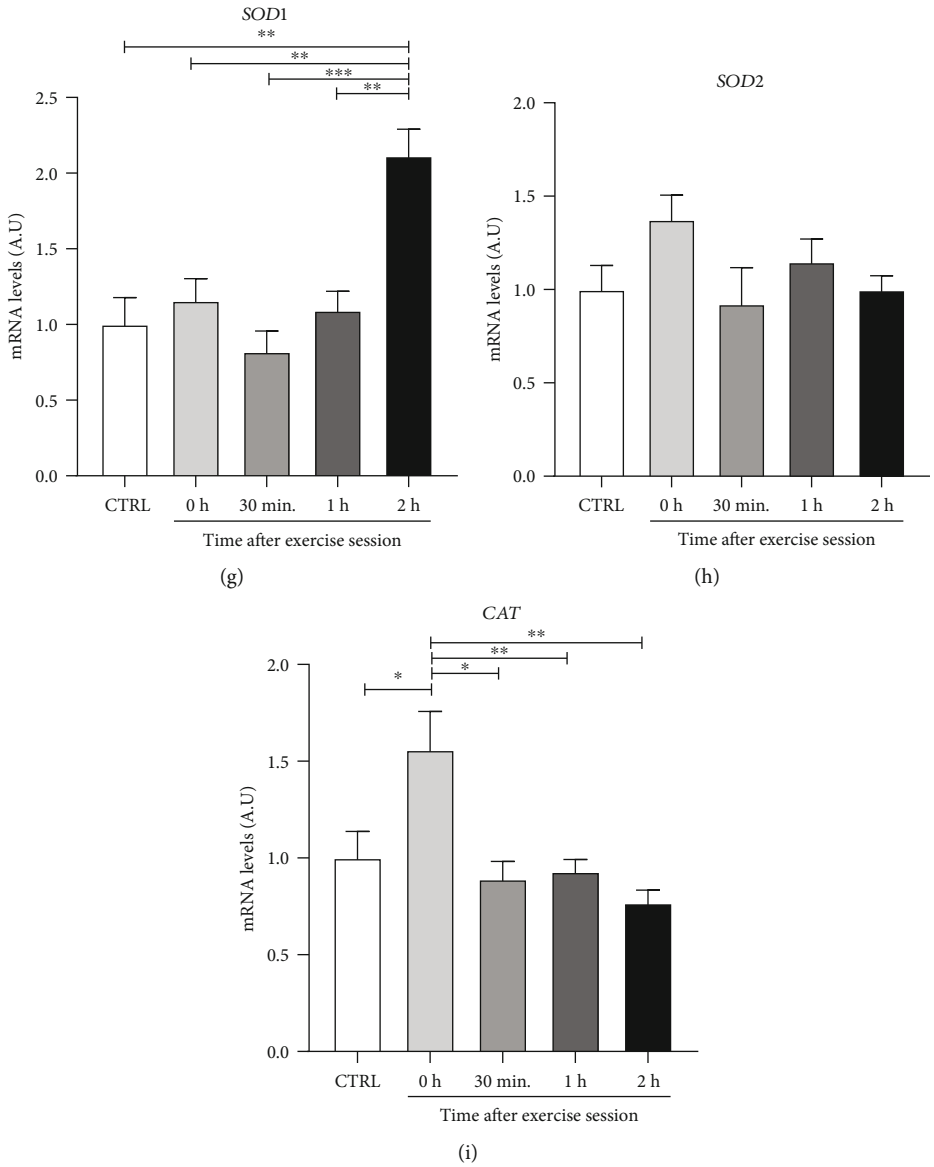

Figure 6: Effect of an acute exercise session on mRNA expression of genes related to antioxidant defense in retroperitoneal WAT. CTRL: control; 0 h; euthanasia immediately after exercise; 30 min: euthanasia 30 minutes after exercise; 1 h: euthanasia 1 hour after exercise; 2 h: euthanasia 2 hours after exercise. (a) NRF2: nuclear erythroid factor related to factor 2; (b) GPX1: glutathione peroxidase 1; (c) GPX2: glutathione peroxidase 2; (d) GCLM: glutamate-cysteine ligase modifying subunits; (e) GCLC1: catalytic subunit 1 glutamate-cysteine ligase; (f) HMOX1: heme oxygenase 1; (g) SOD1: superoxide dismutase 1; (h) SOD2: superoxide dismutase 2; (i) CAT: catalase. Data were expressed as the mean ± standard error of the mean ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

factors that are increased during acute exercise, which was not the aim of the present study.

The increase of mitochondrial respiration is associated with the electron escape of complex I and III, leading to a partial reduction of O_2 forming superoxide anion ($O_2^{\bullet-}$) [52], which increases extramitochondrial H_2O_2 efflux [52]. Although we have found an increase in mitochondrial respiration immediately after exercise, ATP production was reduced. Moreover, an inverse relationship between ATP and ROS production was also observed. Das and Jana (2015) observed higher levels of protein carbonyls in the α-subunit of the F1 complex of skeletal muscle ATP synthase of old mice when compared to their young counterparts. Interestingly, it was associated with a lower activity of this channel in old animals [53]. This is strong evidence of redox-sensitive sites at ATP synthase, and that redox modifications of the ATP synthase α-subunit could play an important role in the regulation of ATP synthesis [54]. Wang et al. (2011) demonstrated in mitochondria isolated from rat hearts that blocking the oxidation of free thiols with N-Ethylmaleimide (NEM, an alkylating reagent) led to increased ATPase enzyme activity. The authors have shown that the difference between the groups treated and not treated with NEM was large (two times), suggesting that the oxidative modification of Cys have a profound effect on the ATPase activity. Still in this study, the authors observed the

presence of a disulfide bond between the ATP synthase α-subunit and the γ-subunit. Nevertheless, the ATPα subunit was identified as the main mitochondrial protein that undergoes oxidative changes induced by ROS, which is associated with reduced activity of the enzyme ATPase [55]. In our findings, we observed that ATP production was reduced immediately after exercise, at the same moment that ROS levels increased. However, from 30 minutes after exercise, ATP production increased, indicating a possible mechanism of ROS modulating mitochondrial ATP production.

ROS availability in a given tissue depends on their production and detoxification. Since our exercise session increased NOX- and mitochondria-derived ROS production, we investigated the activity of the antioxidant enzymes SOD, CAT, and GPX. Nevertheless, no differences among groups were observed concerning SOD and GPx activities. On the other hand, we observed a significant reduction in CAT activity at all-time points after the exercise session. Ping et al. (2015) showed in male mice skeletal muscle a similar response to acute aerobic exercise, with an increase in mitochondrial H_2O_2 production, a reduction in CAT activity up to 90 minutes after exercise, and no differences in SOD activity [56]. CAT is a peroxisomal enzyme that converts H_2O_2 into water and O_2 [57]. Since catalase detoxifies H_2O_2, the decrease in its activity observed after exercise may result in greater availability of ROS.

Our results showed that reactive protein thiol levels were significantly lower immediately after exercise. However, these effects were transient, returning to baseline levels 2 hours after exercise, indicating a recovery from the prooxidant state. Thiols include any organosulfur compound containing the group R-SH in the reduced state (R represents an alkyl group or other organic substituent). The oxidation of thiol groups in cysteine residues often leads to the formation of disulfide bridges, which can importantly impact the tridimensional structure and activity of a protein, for example [58]. Reduced cysteine thiol (Cys-SH) and their oxidized disulfide counterparts are carefully balanced to maintain redox homeostasis in various cellular compartments. Because of that, their levels are related to ROS availability. Moreover, there is a consistent body of evidence showing that transient thiol oxidation is related to redox-sensitive signaling modulation, impacting on cell-signaling proteins, modifying protein kinase activity, and transcription factors [59, 60].

We also observed an increase of lipid peroxidation right after the exercise session, which was sustained after 0.5 and 1 h. Previous reports demonstrated an increase of lipid peroxidation products in the blood after physical exercise [57]. 4-HNE can react with the thiol and amino groups of macromolecules and it is linked to the formation of stable covalent Michael and Schiff base adducts to a wide group of proteins, resulting in protein crosslinking, protein aggregation, inactivation of protein function, and structural perturbation, including increase protein carbonylation [58–60]. Moreover, transient increases in 4-HNE levels have been associated with the activation of Nrf2, increasing mitochondriogenesis, and antioxidant defense [61].

So next, we also evaluated protein carbonyl levels, another marker of oxidation that is formed due to oxidative deamination of lysine and glutamic acid [57]. We observed an increase in protein carbonyl levels immediately and 30 minutes after the end of the exercise session, which returned to basal levels after one hour. These results are in line with the pattern of the most of redox parameters assessed here (increased ROS generation, decreased CAT activity, reduced protein thiol content, and increased 4-HNE) observed immediately after exercise, showing that acute exercise elicited a transitory pro-oxidative state in WAT.

Aerobic exercise in rodent models has consistently been shown to activate Nrf2 signaling in multiple tissues, including skeletal muscle [59], liver [62], and myocardium [63], which leads to the upregulation of endogenous antioxidant gene and protein expressions, one of the physiological adaptations related to the beneficial effect of physical exercise [7]. In basal conditions, NRF2 remains located in the cytoplasm, linked to Kelch-like ECH-associated protein 1 (Keap1), being inactive. The association with Keap1 leads to ubiquitination and, consequently, protease-mediated degradation of NRF2. However, an increase of ROS availability can trigger Keap1 oxidation, decreasing NRF2-Keap1 interaction and, consequently, reducing NRF2 degradation [64]. This process increases NRF2 translocation to the nucleus and its binding to antioxidant response element (ARE), leading to the transcription of more than 200 cytoprotective genes, including NRF2 itself [7]. We observed an increase of NRF2 mRNA levels 1 hour after the exercise session, which suggests that NRF2 signaling was activated in our model, probably due to the transient increase in ROS generation.

Furthermore, one session of exercise was also able to increase mRNA levels of several antioxidant and cytoprotective genes related to NRF2 activation in WAT, including HMOX, SOD1, CAT, and GCLM. The mRNA levels of HMOX1 were higher in the 1 h group. HMOX1 gene codifies hemeoxygenase 1, the enzyme that catalyzes the first and rate-limiting step in heme degradation reaction, producing CO, iron, and biliverdin. HMOX1 is involved in antioxidant anti-inflammatory functions, and it is present at very low levels in most cells and tissues, being upregulated in prooxidant conditions [65]. SOD1 gene expression was also increased after exercise. This enzyme is one of three SOD isoforms, responsible for the dismutation of $O_2^{\bullet-}$ to H_2O_2. CAT gene expression increased immediately after exercise; however, it returned to basal level 30 minutes after the session. CAT gene encodes catalase, a key antioxidant enzyme present in the peroxisome of nearly all aerobic cells that converts H_2O_2 to water and oxygen [66]. Curiously, an acute reduction in GCLM gene expression was lower in the 1 h group in comparison to CTRL and 0 h groups. GCLM is related to the glutathione synthesis from L-cysteine and L-glutamate [67]. The lack of response regarding the expression of GPX1, GPX2, GCLC1, GCLM, and SOD2 was inconsistent with the increase found in other antioxidant genes. The literature is scarce in the description of the effect of acute physical exercise on the dynamics of regulation of cytoprotective genes. Thus, more studies are necessary to elucidate this question.

In the present study, the dynamics of DNA oxidation and DNA damage response after acute exercise were not

evaluated, but this is an important topic that will be addressed in future studies. Moreover, the effect of chronic aerobic exercise on WAT redox homeostasis and DNA damage response of control and obese animals is also a relevant issue that our group intends to evaluate.

5. Conclusion

In conclusion, we demonstrate that one session of aerobic exercise induced a transient prooxidant environment, characterized by a higher NOX activity and extramitochondrial ROS production, decreased CAT activity, and higher levels of biomolecules oxidation. Moreover, mRNA levels of antioxidant and cytoprotective genes were increased by acute exercise, suggesting a cellular response to the transient ROS exposure.

Data Availability

All data used to 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.

Acknowledgments

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Research Article

A Short-Term Resistance Training Circuit Improved Antioxidants in Sedentary Adults with Down Syndrome

M. Rosety-Rodriguez,[1] M. Bernardi,[2] S. Elosegui,[1] I. Rosety,[1] A. J. Diaz,[3] M. A. Rosety,[4] F. Brenes,[1] A. Oliva-Pascual-Vaca,[5] J. R. Alvero-Cruz,[6] and F. J. Ordonez[7]

[1]*School of Medicine, University of Cadiz, Cadiz, Spain*
[2]*School of Specialty in Sports Medicine and Physical Exercise, Department of Physiology and Pharmacology "V. Erspamer", Sapienza University of Rome, Rome, Italy*
[3]*School of Nursing, University of Cadiz, Cadiz, Spain*
[4]*School of Sports Sciences, University of Cadiz, Cadiz, Spain*
[5]*School of Physiotherapy, University of Seville, Seville, Spain*
[6]*Department of Physiology, Histology, Pathological Anatomy and Sports Physical Education, University of Malaga, Malaga, Spain*
[7]*School of Sports Medicine, University of Cadiz, Cadiz, Spain*

Correspondence should be addressed to F. J. Ordonez; franciscojavier.ordonez@uca.es

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Previous studies have found aerobic training improved oxidative damage in people with Down syndrome (DS). However, there is a lack of information regarding the influence of resistance training on redox imbalance in this population. Accordingly, this study was conducted to determine the effect of resistance training (RT) on antioxidant defence system in sedentary adults with DS. Thirty-six male adults with DS were recruited through different community support groups. Eighteen were randomly assigned to perform a circuit RT program with 6 stations, 3 days/week for 12 weeks. Plasma total antioxidant status (TAS), reduced glutathione (GHS), ascorbate, serum α-tocopherol, and erythrocyte glutathione reductase activity were assessed. Plasma malondialdehyde (MDA) and carbonyl groups (CG) were assessed as markers of oxidative damage. Muscle strength was also measured. Dynamic torque of knee extensors and flexors as well as maximal handgrip strength was significantly improved after the completion of the training program. Plasma levels of TAS and erythrocyte glutathione reductase (GR) activity were significantly increased. Conversely, MDA and CG levels were significantly reduced. It was concluded RT improved antioxidant defence system and reduced oxidative damage in adults with DS. Further, long-term studies are required to determine whether the increased antioxidant system may improve clinical outcomes of adults with DS.

1. Introduction

In the last decades, life expectancy of people with Down's syndrome (DS) has increased considerably, now exceeding 60 years [1]. Accordingly, it has resulted in extended periods of adulthood requiring specialist care to ensure personal well-being [2]. In this respect, the most common comorbid diagnoses among people with DS, such as early aging, dementia, and immunodeficiency, have been strongly associated with oxidative damage [3]. This is of particular interest given that people with DS present a higher oxidative stress because of the overexpression of genes on chromosome-21 [4].

In a recent study, Oppewal et al. [5] reported that physical fitness has been independently associated with survival in adults with intellectual disability (ID). In addition, increased exercise may have positive changes on memory and other cognitive functions [6].

Consequently, regular exercise should be encouraged as key part of care and support for this group. In this respect, previous studies have found that aerobic training programs

reduced oxidative damage in DS by increasing antioxidant enzyme activities [7–9].

However, there is a lack of information regarding the influence of resistance training on redox imbalance in people with DS. In this respect, muscle hypotonia has been traditionally considered a major barrier to RT for people with DS [10]. It is also generally accepted that RT involves an inherent risk of musculoskeletal injury that could be higher for people with ID because of their preexisting disability [11]. The reasons already mentioned may contribute, at least in part, to hinder health-care providers to encourage RT for people with DS. Fortunately, in a previous study, our research group reported resistance training was safe and effective in order to reduce systemic low-grade inflammation in adults with DS [12] and without DS [13].

For the reasons already mentioned, this study was conducted to ascertain the effects of a short-term circuit resistance training on antioxidant defence system and markers of oxidative damage in sedentary adults with DS.

2. Methods

2.1. Participants. A total of thirty-six adults with DS (28.1 ± 3.3 years) were recruited for the current interventional study through different community support groups for people with ID. They had an intelligence quotient (IQ) range of 60–69, determined by Stanford-Binet Scale, being diagnosed as having mild ID. Participants were excluded from the study if they meet any of the following criteria: (1) atlantoaxial instability, (2) cardiovascular diseases (congenital heart disease, etc.), (3) metabolic disorders (diabetes, thyroid disease, etc.), (4) toxic habits (smoking or alcohol), (5) nutritional supplements, (6) participation in a training program in the 6 months prior to study entry, and (7) not completing at least 90% of the training sessions. The adequacy of sample size was tested using the statistical software Granmo *v7*.12 (IMIM, BCN, Spain) with an accepted two-sided alpha risk of 0.05 and a beta risk of 0.2. Lastly, a loss to follow-up rate of 10% was also estimated.

Eighteen were randomly assigned to the experimental group using a concealed method. The control group included 18 age, sex, and BMI matched adults with DS who did not take part in any training program (Table 1).

2.2. Intervention Program. The intervention consisted of a supervised circuit resistance training, 3 days per week for 12 weeks (Table 2).

This training was circularly performed in 6 stations: arm curl, leg extension, seated row, leg curl, triceps extension, and leg press. Each training session started and finished with a warming-up and cooling-down period of 5-10 minutes during which muscle stretching exercises were performed [14]. Furthermore, training sessions were in small groups (6 participants) and were supervised by experienced physical therapists to ensure that participants used the correct technique and intensity (ratio 1 monitor/2 participants).

It should be pointed out that before starting training program, participants included in the intervention group underwent a pretraining session to be familiar with resistance exercises as well as to perform the 8-repetition-maximum (8RM) test per each exercise [15].

Table 1: General characteristics at baseline of both the intervention ($n = 18$) and control ($n = 18$) groups.

	Intervention	Controls	p value
Age (years)	28.4 (23.7-33.6)	27.8 (23.2-32.9)	>0.05[a]
Weight (kg)	71.1 (66.0-76.6)	69.8 (64.8-75.1)	>0.05[a]
BMI (kg/m^2)	31.4 (27.9-36.1)	30.8 (27.1-35.7)	>0.05[a]
LDL-C (mg/dl)	124.6 (7)	118.1 (8.4)	>0.05[b]
HDL-C (mg/dl)	48.7 (4.4)	51.2 (4.8)	>0.05[b]
TGL (mg/dl)	138.3 (8.2)	130.3 (9.4)	>0.05[b]
Energy intake (kcal)	1811 (203)	1786 (194)	>0.05[b]
Vitamin C (mg/d)	82.2 (23.7)	77.4 (22.0)	>0.05[b]
Vitamin E (mg/d)	9.6 (2.1)	9.3 (1.9)	>0.05[b]

BMI: body mass index; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TGL: triglycerides. Results were expressed as mean (SD) or median (IQR). [a]Mann–Whitney U test for change scores. [b]ANOVA test for change scores.

Table 2: Resistance circuit training, comprised of 6 stations, performed by participants in the intervention group.

	1-2 weeks	3-4 weeks	5-6 weeks	7-8 weeks	9-10 weeks	11-12 weeks
Load	40%	40%	45%	45%	50%	50%
Series	2	2	2	2	2	2
Rep.	10	10	10	10	8	8
Rest	90	90	90	90	90	90

Load: expressed as percentage of 8 repetition-maximum (8RM) test. Rep: number of repetitions. Rest: resting periods between stations expressed in seconds.

2.3. Nutritional Intake Record. To control the potential confounding effect of diet, parents were carefully instructed to avoid quantitative or qualitative differences. Furthermore, they were asked to complete a food consumption frequency questionnaire for three days (2 weekdays and 1 weekend day). Energy and nutrient intake were calculated using a specific software (VD-FEN 2.1, Madrid, Spain) based on updated Spanish food composition tables [16].

No significant difference was found between the intervention and control groups when assessing energy intake (1811 ± 203 vs. 1786 ± 194 kcal; $p = 0.41$). Furthermore, mean daily vitamin intake showed no significant differences (9.6 ± 2.1 vs. 9.3 ± 1.9 mg/d vitamin E $p = 0.71$; 82.2 ± 23.7 vs. 77.4 ± 22.0 mg/d vitamin C $p = 0.58$).

2.4. Outcome Measurements. All outcomes at individual level were assessed firstly at baseline and secondly 72 h after the end of the intervention.

Blood samples were obtained from antecubital vein puncture and collected in heparinized tubes. The whole blood was centrifuged at 3000 rpm for 20 minutes in a clinical centrifuge. Plasma total antioxidant status (TAS) was determined spectrophotometrically on a Hitachi 902 Autoanalyzer (Roche,

Alameda, CA) by commercial kits (Randox, Crumlin, UK). Similarly, reduced glutathione (GSH) level was determined colorimetrically at 412 nm following reaction with DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) according to the instructions of manufacturer.

The erythrocytes remaining after the removal of the plasma were washed three times with 310 mM isotonic Tris-HCl buffer (pH 7.4). Hemolysis was carried out by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes which contained 20 mM hypotonic Tris-HCl buffer (pH 7.2). Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured by using the xanthine oxidase-cytochrome c method according to McCord and Fridovich's method, with slight modifications [17]. In this line, one unit represented the activity that inhibited cytochrome c reduction by 50%.

Glutathione reductase (GR, EC 1.6.4.2.) activity was measured by following the decrease in absorbance due to the oxidation of NADPH as described by Goldberg and Spooner [18]. In this respect, one activity unit was defined as 1 μmol NADP (NADPH) formed $min^{-1} l^{-1}$ haemolysate.

Finally, plasma ascorbate was analyzed by paired-ion, reversed-phase HPLC as previously described [19]. And serum α-tocopherol was assessed by reversed-phase HPLC, using a C18 column and a photodiode array detector [20].

Similarly, levels of MDA were determined, based on the hydrolysis of lipoperoxides in plasma, using HPLC in reverse phase and quantified at 532 nm as previously described [21]. Results were expressed in μmol/l.

Plasma levels of carbonyl groups were performed using an ab126287 Protein Carbonyl Content Assay kit (Abcam, Cambridge, UK). Absorbance was measured in an ELISA plate reader at 375 nm according to the instructions of manufacturer. Results were expressed as nmol of protein carbonyl group formation per mg of total protein. In this respect, the total protein content of plasma samples was also determined using commercial kits (Pierce BCA Protein Assay kit [23225], Thermo Fisher scientific, Waltham, MA, USA). Lastly, it should be pointed out all samples were analyzed in triplicate.

The Jamar handgrip electronic dynamometer (Bolingbrook, Illinois, US) was used to assess maximal handgrip strength of the dominant hand, defined as the one preferred for daily activities. It should be pointed out the one handle position was used as recommended for people with small hand size [22]. The standard testing position, approved by the American Society of Hand Therapists, was used [23].

The measurements of dynamic torques produced by knee flexors and extensors at an angular velocity of 90°/s were conducted using the motor-driven dynamometer Technogym-REV 9000 (Technogym Spa, Gambettola, Italy). All tests, conducted by the same investigator, were performed in a sitting position with hip flexed at 90°. During trials, restraining belts were placed around the chest and abdomen to stabilize the body and, again, to minimize contractions with other muscles. Furthermore, the lever arm was attached to the midline, and its axis of rotation aligned with the anatomic axis of knee rotation. Prior to every testing, participants performed a standardized warm-up on a stationary bicycle at a comfortable pace followed by light stretching leg exercises. Participants were asked to exert the maximal force over the full range of motion.

For both, the Jamar and Technogym-Rev 9000 dynamometers, three maximal attempts, separated each one by 90-second (maximal handgrip) and 20 minute (peak torque) resting periods, were given by each subject. The highest value was considered for further analysis. Verbal encouragement was afforded to ensure maximal efforts. Furthermore, all participants ($n = 36$) underwent a preliminary session to be familiar with the correct use of both dynamometers [24].

2.5. Ethics and Statistics. It should be pointed out that the current protocol complied with the Declaration of Helsinki (2008). Written informed consent was obtained from all their parents or legal representatives. Further, the current protocol was approved by an Institutional Ethics Committee (Protocol number 29-076/2018).

The results were expressed as a mean and standard deviation (SD) or median and interquartile range (IQR). The Shapiro-Wilk test was used to assess whether data were normally distributed. To compare the mean values, a one-way analysis of variance (ANOVA) with post hoc Bonferroni correction to account for multiple tests was used. Lastly, Cohen's d statistics were used for determining mean effect sizes as follows: small $d \geq 0.2$ and <0.5; medium $d \geq 0.5$ and <0.8; large $d \geq 0.8$. Mann–Whitney U test was used for significance of difference between intervention and control groups when the data were not normally distributed. Pearson's correlation coefficient (r) was used to determine potential associations among tested parameters. For all tests, statistical significance was set at an alpha level of 0.05.

3. Results

When compared to baseline results, peak torques at an angular velocity of 90°/s of knee extensors (48.3 ± 2.8 vs. 55.1 ± 3.3 Nm; $p = 0.22$) and flexors (28.1 ± 1.9 vs. 32.1 ± 2.0 Nm; $p = 0.412$) were significantly improved in the intervention group. Angles at peak torque value for knee extensors and flexors were 57.8 ± 5.2° and 42.0 ± 4.1°, respectively. Furthermore, maximal handgrip strength was also significantly greater (28.3 ± 7.2 vs. 30.8 ± 7.4 kg; $p = 0.36$).

Regarding antioxidants, resistance training significantly increased plasma TAS (0.38 ± 0.07 vs. 0.45 ± 0.05 nmol/l; $p < 0.001$). In a more detailed way, erythrocyte GR activity (11.8 ± 2.6 vs. 13.2 ± 2.7 mg/ml; $p = 0.022$) and plasma levels of reduced glutathione (8.3 ± 0.8 vs. 9.6 ± 0.9 U/gHb; $p = 0.031$) were significantly increased after the completion of the resistance circuit training. Conversely, no significant changes were found in plasma ascorbate ($p > 0.05$) and serum α-tocopherol ($p > 0.05$).

It was also found that both markers of oxidative damage, MDA (1.76 ± 0.61 vs. 1.38 ± 0.50 μmol/l; $p = 0.011$) and carbonyl groups (7.82 ± 2.90 vs. 6.19 ± 2.38 nmol/mg; $p = 0.037$), were significantly reduced in the intervention group.

Finally, a weak but significant correlation was found between GR activity and maximal handgrip strength ($r = 0.32$; $p = 0.044$) in the intervention group. No changes

Table 3: Effects of resistance training on antioxidant defence system and markers of oxidative damage in sedentary young adults with Down syndrome.

	Exercising group		Control group		
	Pretest	Posttest	Baseline	Final	Cohen's d
TAS (nmol/l)	0.38 ± 0.07	0.45 ± 0.05[a,b]	0.36 ± 0.08	0.37 ± 0.07	1.14
SOD (U/gHb)	436.6 ± 25.9	449.8 ± 27.8	441.6 ± 26.1	445.2 ± 26.6	0,17
GR (U/gHb)	8.3 ± 0.8	9.6 ± 0.9[a,b]	8.1 ± 1.0	8.2 ± 0.9	1.33
GSH (mg/ml)	11.8 ± 2.6	13.2 ± 2.7[a,b]	11.4 ± 2.5	11.2 ± 2.4	0.83
Vit. E (μmol/l)	13.5 ± 2.4	13.9 ± 2.5	13.3 ± 2.7	13.4 ± 2.6	0.19
Vit. C (μmol/l)	60.7 ± 9.2	61.1 ± 9.1	59.3 ± 9.4	59.1 ± 9.5	0.16
MDA (μmol/l)	1.76 ± 0.64	1.38 ± 0.50[a,b]	1.69 ± 0.68	1.72 ± 0.65	1.04
CG (nmol/mg)	7.82 ± 2.90	6.19 ± 2.38[a,b]	7.60 ± 3.08	7.63 ± 3.01	0.79

TAS: total antioxidant status; superoxide dismutase activity; GR: glutathione reductase activity; GSH: reduced glutathione; Vit E: vitamin E or α-tocopherol; Vit. C: vitamin C or ascorbate; MDA: malondialdehyde; CG: carbonyl groups. Results were expressed as mean ± SD. [a]$p < 0.05$ versus pretest; [b]$p < 0.05$ versus control group (final).

were found in any assessed outcome in the control group. These results are summarized in Table 3.

4. Discussion

The present study was the first to evaluate the influence of RT on antioxidant defence system in adults with DS. As was hypothesized, strength training significantly increased total antioxidant status. This effect may be explained, at least in part, by increasing the activity of antioxidant enzymes such as erythrocyte GR and, as a consequence, plasma levels of reduced glutathione. In contrast to single bouts of exercise [25], regular exercise induced an increased antioxidant enzyme activity that was able to reduce markers of oxidative damage to lipids and proteins in the current study.

Similar results regarding the antioxidant effect of aerobic training programs have been reported in people with intellectual disability. In a more detailed way, the improvement found in antioxidant enzyme activity was capable of reducing oxidative damage in this population after the completion of the training program [7–9]. In agreement with Nocella et al. [26], future intervention programs that combines training and antioxidant supplementation are necessary considering the latter may increase nonenzymatic antioxidant system.

On the other hand, RT has received much less attention on this topic. Not only in studies focused on disabled people [12] but also on general population, in spite of published results were promising [27, 28]. In this respect, Gambassi et al. [27] found that an 8-week dynamic resistance training protocol improved oxidative damage in stroke survivors. Similarly, Bachi et al. [28] reported a mixed intervention program that combined aerobic and resistance training (3 times/week for 18 months) reduced plasma oxidative stress in sedentary elderly adults. Conversely, Medeiros et al. [29] reported that an intervention based on RT reduced protein oxidative damage (expressed as carbonyl groups) but increased oxidative damage to lipids (expressed as TBARS) in obese adults. This finding could be explained, at least in part, considering that TBARS assay is nonspecific because it can detect aldehydes other than MDA [30].

Another challenge of the present study was to identify a significant correlation between GR activity and maximal handgrip strength. These results were in agreement with previous studies that found significant associations between increased oxidative stress and muscle mass and strength in community-dwelling older adults [31, 32].

Regarding the functional assessment of participants, the present intervention program significantly improved muscle strength in upper and lower limbs. Conversely, regular participation in basketball without the application of any resistance training program just improved isometric and isokinetic peak torques in the lower limbs in adults with intellectual disability [33]. These findings are of particular interest considering the positive effects of muscle strength on functional tasks of daily living and employability in young adults with DS [34, 35]. In addition, it may finally give them the confidence to continue exercising after the intervention finished [36].

Despite several benefits associated with physical activity, it is also generally accepted that RT involves an inherent risk of musculoskeletal injury that could be higher for people with ID [11]. Fortunately, neither sport-related injuries nor dropouts were reported during the whole experience suggesting not only the effectiveness but also the safety of the present circuit training program.

Finally, the present study had some limitations. A major weakness was the relatively short duration of the exercise intervention in that there was no follow-up to determine whether the positive effects induced by resistance training were maintained. Furthermore, the use of weight lifting machines may limit the reproducibility of this study in case exercise equipment is not available. Accordingly, future studies focused on circuits that utilize body weight or free-weight exercises that could be conducted at both homes or nursing homes are also required to guarantee its reproducibility. In fact, these intervention programs would be of great interest in the current confinement provoked by

COVID-19 pandemic that includes restrictions of access to indoor sport centres.

Strengths of the current study included the excellent adherence rate as well as the homogeneous and large sample size. Conversely, many studies that have been focused on the influence of regular exercise on people with ID have recruited mixed (male and female) groups in order to increase sample size with the aim of strengthening research design [6, 37]. In addition, some studies have recruited participants with intellectual disability matched for intelligence quotient but having different diagnoses [38]. Furthermore, the presence of a control group consisting of age, IQ, and sex matched individuals with DS may reduce the recruitment bias of nondisabled controls.

In conclusion, resistance training improved antioxidant defence system in male adults with DS by increasing antioxidant enzyme activity. In addition, it reduced plasma levels of oxidative damage to lipids and proteins. Further, long-term follow-up studies are required to determine whether the increased antioxidant system induced by regular resistance training may improve clinical outcomes of adults with DS.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Healthy Lifestyle Recommendations: Do the Beneficial Effects Originate from NAD^+ Amount at the Cellular Level?

Borut Poljsak,[1] **Vito Kovač**,[1] **and Irina Milisav**[1,2]

[1]*Laboratory of Oxidative Stress Research, Faculty of Health Sciences, University of Ljubljana, Zdravstvena pot 5, Ljubljana, Slovenia*
[2]*Faculty of Medicine, Institute of Pathophysiology, University of Ljubljana, Zaloska 4, Ljubljana, Slovenia*

Correspondence should be addressed to Irina Milisav; irina.milisav@mf.uni-lj.si

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In this review, we describe the role of oxidized forms of nicotinamide adenine dinucleotide (NAD^+) as a molecule central to health benefits as the result from observing selected healthy lifestyle recommendations. Namely, NAD^+ level can be regulated by lifestyle and nutrition approaches such as fasting, caloric restriction, sports activity, low glucose availability, and heat shocks. NAD^+ is reduced with age at a cellular, tissue, and organismal level due to inflammation, defect in NAMPT-mediated NAD^+ biosynthesis, and the PARP-mediated NAD^+ depletion. This leads to a decrease in cellular energy production and DNA repair and modifies genomic signalling leading to an increased incidence of chronic diseases and ageing. By implementing healthy lifestyle approaches, endogenous intracellular NAD^+ levels can be increased, which explains the molecular mechanisms underlying health benefits at the organismal level. Namely, adherence to here presented healthy lifestyle approaches is correlated with an extended life expectancy free of major chronic diseases.

1. Introduction

Fasting, caloric restriction, sports activity, low glucose availability, and heat shocks are lifestyle and nutrition approaches that influence NAD^+ levels [1–6]. Deficiency in NAMPT-mediated NAD^+ biosynthesis, increased inflammation, and the PARP-mediated NAD^+ depletion are causes of reduced NAD^+ levels with age at a cellular, tissue, and organismal level [7, 8].

Coenzyme nicotinamide adenine dinucleotide (NAD^+), which contains two covalently joined mononucleotides (nicotinamide mononucleotide or NMN, and AMP) [9], has an important role in an energy metabolism like mitochondrial electron transport, glycolysis, and citric acid cycle [10] in order to generate adenosine triphosphate (ATP) [11]. NAD^+ is also a rate-limiting substrate for many signalling enzymes such as sirtuin (SIRT) proteins SIRT1 and SIRT3, the poly (ADP-ribose) polymerase (PARP) proteins PARP1 and PARP2, a COOH-terminal binding protein (CtBP), cyclic ADP-ribose (ADPR) synthetases CD38 and CD157, and many other NAD^+-dependent enzymes. These enzymes are involved in important cellular processes, like DNA repair, stress response, genomic stability, chromatin remodelling, circadian rhythm regulation, cell cycle progression, insulin secretion and sensitivity, and expression of the inflammatory cytokines, thus translating changes in energy status into metabolic adaptations [12]. NAD^+ is recycling during ATP formation in processes of glycolysis, beta-oxidation, Krebs cycle, and electron transport in cytosol and mitochondria and shifts between reduced and oxidized forms as required for the continuous flow of electrons across the metabolic pathways. Therefore, the NAD^+ molecule is conserved during these processes. On the other hand, the NAD^+ is consumed during cellular signalling, in adenosine diphosphate (ADP)-ribosyl transfer reactions, by poly-ADP-ribose polymerases (PARPs), sirtuin deacetylases (Sirtuins), and the cluster of differentiation 38 (CD38), i.e., the nicotinamide (NAM) unit is separated. NAD^+ half-life is between 1–2 h in the cytoplasm and nucleus and approximately 8 h in the mitochondria [13] and can be salvaged and reused by three pathways: (1) *de novo* synthesis (from L-tryptophan), (2) Preiss-Handler pathway (from nicotinic acid or nicotinic

acid ribose), and (3) salvage pathway (from niacinamide/nicotinamide, nicotinamide riboside, and nicotinamide mononucleotide) [9, 14–18]. NAD^+ is mainly produced by the NAD^+ salvage pathway where nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme, converting NMN into NAD^+ [19–21]. NAMPT regulates processes related to the pathological processes of obesity and a type 2 diabetes mellitus by influencing lipid and glucose metabolism, insulin resistance, the oxidative stress response, apoptosis, and inflammation [22, 23].

The NAD^+/NADH ratio influences also the reactive oxygen species (ROS) and oxidative stress formation through regulation of intracellular ATP production, different metabolic enzymes, and redox state. An increase of NAD^+ and/or NAD^+/NADH ratio can increase cell defence, can induce DNA repair and apoptosis through activation of PARPs and sirtuins, and thus plays an important role in the prevention of cancerogenesis and many other diseases [14, 24]. For example, cellular NAD^+/NADH ratio regulates SIRT1 enzymatic activity, which further regulates a number of target proteins [25], such as FOXO family of transcription factors [26–28], p53 [29, 30], PGC-1a [31, 32], and NF-kB [33–35]. While chronic diseases and ageing are related to decreased NAD^+ levels [16, 36, 37], different lifestyle factors have been found that ameliorate NAD^+ bioavailability, which positively affects SIRT stimulation and subsequent PGC-1α and FOXO1 expression, leading to mitochondrial changes and metabolic adaptations (Figure 1) [38]. Increased available cellular energy, improved stem cell and mitochondrial function, DNA repair [39], telomere maintenance [40], and enhanced metabolic activity are prerequisites for effective health span and life span [41, 42] as demonstrated by studies where NAD^+ levels were intentionally increased [23, 43–48].

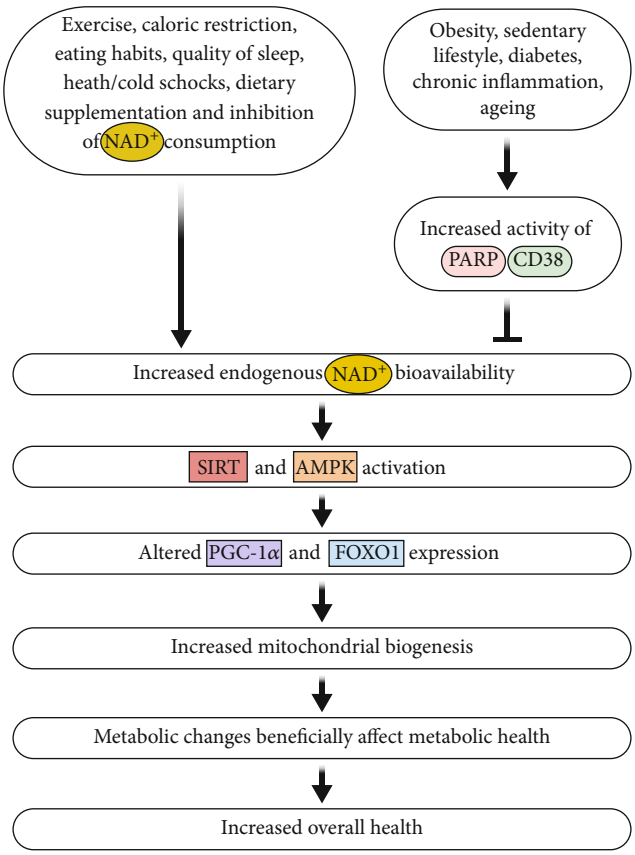

Figure 1: Health benefits as a result of implementing approaches to increase NAD^+ bioavailability.

2. Caloric Restriction, Eating Habits, and NAD^+ Levels

A well-balanced diet in macro- and micronutrients represents a basis for health and well-being. Limited calorie intake continues to be the strategy supported by the greatest evidence for ensuring increased lifespan and health [49]. In different model organisms, a significant increase in lifespan was reported if calories were restricted between 25–60% relative to normally fed control [50, 51]. How is caloric restriction connected with NAD^+ levels? CR stimulates the NAD^+ salvage pathway leading to increased NAD^+ bioavailability by activating the expression of NAMPT, which triggers the NAD^+ salvage pathway by transforming nicotinamide (NAM) to NAD^+ [52]. Caloric restriction increases NAD^+ levels, while lowers NADH levels and activates sirtuins [53]. For example, caloric restriction extends the yeast's life span by lowering the level of NADH, since NADH is a competitive inhibitor of Sir2 [54]. Thus, activation of sirtuins with a sufficient amount of bioavailable NAD^+ is a necessary condition for the life-span extension provided by CR [55, 56]. Specifically, Sirt1 regulates CR by detecting intracellular low energy levels and provoking physiological changes relevant to health and longevity [57]. On the other hand, inactivation of SIRT1 results in the prevention of CR-mediated lifespan extension [58].

Studies on caloric restriction revealed that it is more important to improve the ratio between NAD^+ and NADH than to raise the overall amount of cellular NAD^+ [59]. Namely, caloric restriction reduces NADH amount more than it influences the NAD^+ levels, at least in yeast [54, 60]. It seems that lowering NADH is an important factor responsible for the increased activity of the NAD^+-consuming enzymes, as NADH is an inhibitor of Sirtuins and PARPs [54].

Besides by caloric restriction, NAD^+ levels can be increased with food and commercially available supplements. Ingestion of the amino acid tryptophan or forms of vitamin B3 (niacin, nicotinic acid, niacinamide) as well as nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), and nicotinic acid riboside (NaR) stimulates the formation of NAD^+ [61–64]. Daily requirements for NAD^+ synthesis can be obtained either with dietary tryptophan or with around 15 mg/d of daily niacin, a collective term for nicotinic acid (NA) and nicotinamide (NAM) [61], which can be found in meat, fish, and dairy products [65].

Small-scale human clinical studies have shown that NAD^+ boosters such as NMN, NR, and niacin can increase the levels of NAD^+ in volunteers and are relatively safe for human consumption [6, 66–72]. Most of the side effects reported during treatment with NAM, NR, and NMN are

minor (e.g., diarrhea, nausea, rashes, hot flashes, cramps in the legs, erythema) and occur relatively rarely [73, 74]. Increased acetylcarnitine concentrations in skeletal muscle and minor changes in body composition and sleeping metabolic rate were reported in the recent study on NR supplementation in healthy obese humans [75]. The evidence for assessing the health risk is still limited, and long-term exposure to NAD^+ booster (NR, NMN) has not yet been investigated in human clinical trials or human clinical trials are not yet completed. In addition, there is insufficient data on increasing the levels of NAD^+ in various clinical disorders.

As data for some newly discovered NAD^+ precursor forms are scarce, NAD^+ supplements should be tested in a manner similar to drugs in development [72]. Niacin equivalents/precursors are found in animal and plant foods, mainly in the form of NA and NAM. Additionally, recently discovered NAD^+ intermediates, such as NMN and NR, are also in foods, like cucumber, cabbage, and immature soybeans. Broccoli has 0.25–1.88 mg of NMN per 100 g, avocado and tomato 0.26–1.60 mg/100 g. Much less NMN can be found also in raw beef and shrimp (0.06–0.42 mg/100 g) [45] as well as human and cow milk at micromolar concentrations [76, 77]. NAD^+ biosynthesis can be increased by direct activation of NAD^+ biosynthetic enzymes by several AMPK and NAMPT activators, like nonflavonoid polyphenol resveratrol, metformin, 5-aminoimidazole-4-carboxamide ribonucleotide, P7C3, leucine, epigallocatechin gallate, and proanthocyanidins [78–86]. CD38, its homologue CD157, and PARP-1 inhibitors could additionally increase NAD^+ availability; however, they are registered as medical drugs for cancer treatment [24], thus beyond the scope of this review.

3. Eating Habits

NAD^+/sirtuin pathway could be influenced also with nutritional approaches, e.g., eating habits. At what time and what and how much food we eat influence intracellular NAD^+ bioavailability by altering electron transport in mitochondria. For example, a high-fat/sugar diet causes energy overload, culminating in reduced NAD^+/NADH ratio [87] and decreases NAD^+ levels [23, 63]. Also, a low AMP/ATP ratio causes a decrease in NAD^+ or NAD^+/NADH, in situations when enormous amounts of calorically rich food (lipids and/or carbohydrates) are eaten. This additionally leads to elevated blood sugar and insulin levels, increased NADH/-NAD ratio, and increased formation of ROS, which triggers the postprandial oxidative stress and oxidative damage [88–91]. Large amounts of electrons from sugars enter the mitochondria after a large portion of food that generates more superoxide at complex 1 (NADH: ubiquinone oxidoreductase) and complex III (ubiquinol: cytochrome c oxidoreductase) [92]. Efficient electron flow and avoidance of electron leaks (superoxide formation) can be achieved if ATP is regularly consumed; for example, by moderate sport activity or any kind of physical work. This increases the AMP/ATP ratio and NAD^+ availability [87, 93, 94]. The link between the metabolism and NAD^+ is further strengthened by observations that besides overnutrition, tissue NAD^+ levels decrease also with high-fat diets and obesity [23, 63, 95–98]. Rappou et al. [99] compared SIRT1, SIRT3, SIRT7, and NAMPT expressions and total PARP activity in lean and obese subjects. Results indicated lower sirtuins and NAMPT expressions and increased total PARP activity in obese compared to lean subjects. After a moderate weight loss, SIRT1 and NAMPT expressions increased while PARP activity significantly decreased in subjects upon the weight loss. Similar results were obtained in healthy men during lipid overfeeding [100]. Other studies observed that obesity is associated with low NAD^+ levels or SIRT pathway expression [101]. On the other hand, supplementation with NAD^+ precursors or intermediates activates sirtuins and oxidative metabolism resulting in the protection against high-fat diet-induced obesity [63], improved glucose tolerance and hepatic insulin sensitivity [23], and lipid metabolism [45].

A high-fat caloric diet induces obesity through the protein CD38, which is a regulator of body weight and an NAD^+ consumer [102]. Mice deficient in CD38 are protected against the high-fat diet-induced obesity due to boosted metabolic rate in part via a NAD-dependent stimulation of SIRT-PGC1alpha axis [102]. Adipose tissue elevates the expression of CD38 and inflammation-related genes in obese people [103, 104]. In line, low expression of CD38 protected against obesity when fed a high-fat diet in animals [102, 105].

NAD^+ level is not only nutritionally controlled, but it depends also on the sports activities and other lifestyle factors.

4. Exercise and NAD^+ Levels

Physical activity and exercise, as part of a healthy lifestyle, have a significant impact on health outcomes, including improved motor skills, healthy bones, enhanced aerobic fitness, efficient heart and lung function, improved cardiovascular health, lowered risk of stroke, certain types of cancer and diabetes, improved metabolic flexibility and mitochondrial function, and a positive effect on cognitive function and mental health—including on depressive symptom improvement and anxiety- or stress-related disease [38, 106–108].

How does sports activity affect NAD^+ levels? Aerobic exercise training or any kind of exercise/sports activity increases the amount of NAD^+ due to the induction of skeletal muscle's NAMPT expression that was shown in rodent and human studies [109–111]. Namely, in human skeletal muscle, exercise training reverses the age-dependent decline of NAD^+ by stimulating the NAD^+ salvage pathway, in which nicotinamide NAMPT is a rate-limiting enzyme [112]. Exercise and aerobic sports activity increases the amount of NAD^+ due to the induction of skeletal muscle's NAMPT expression [109] and reverses the age-dependent decline of NAD^+ by stimulating the NAD^+ salvage pathway [112] through the 5′ AMP-activated protein kinase (AMPK) pathways [4].

NAD^+ has an important role in the generation of intracellular ATP, which is required for exercise and sports activities. On the other hand, as already mentioned, ATP production in mitochondria represents the main source of

free radical generation. The reduction state of complex I in mitochondria depends strongly on the NAD^+ and NADH levels. Ameliorating the NAD^+/NADH ratio by elevated ATP consumption (e.g., sports activity) or decreased ATP production (e.g., intermitted fasting, consumption of small portions of food, and CR) regulates the magnitude of superoxide-generation from the transfer of electrons to molecular oxygen at mitochondrial complexes I and III and can thus ameliorate the intensity of oxidative damage [113]. Increased demand for energy during the exercise is sensed by the cell and activates AMPK, which can modulate NAD^+ bioavailability [38]. Both exercise and caloric restriction trigger the metabolic stress that follows by adaptation by inducing NAMPT expression through the AMPK [4, 109, 114] resulting in increased NAD^+ levels available for sirtuins and PARPs.

A recent study by de Guia et al. revealed that different exercise training methods reverse the age-dependent decline in NAD^+ salvage capacity in the human skeletal muscle [112]. Namely, both aerobic and resistance exercise training increased NAMPT levels in young and older individuals. In aged rats, exercise training also increased NAD^+, NAMPT levels, and SIRT1 activity [111] and accelerates the *de novo* biosynthesis of NAD^+ from L-tryptophan [115].

The important function of NAD^+ during sports activity is its role as a hydrogen/electron transfer molecule for adenosine triphosphate (ATP) production and mitochondrial biogenesis in muscle cells [116]. Additionally, sports activity increases the NAD^+ amount also at the systemic level [117] that results in health benefits at the organismal level due to the NAD^+ role in multiple and diverse cellular processes, in addition to redox reactions, such as deacetylation and ADP-ribosylation [116]. During the intense sports activity, ATP is consumed; thus, the need for NADH as the electron donor increases, which in the end results in the boosted formation of oxidised NAD^+ and decreased NADH, i.e., an improved NAD^+/NADH ratio. The total amount of NAD^+ is not significantly changed during the redox reaction; however, the *NAD^+/NADH* (and NADP to NADPH) ratio is changed in favour of NAD^+ [61], which activates sirtuins, PARPs, CD38, and other NAD^+-consuming reactions. Since NAD^+-consuming enzymes intervene in many crucial cellular processes, many healthy processes at the organismal level are enhanced by the implementation of exercise and sports activity.

Surprisingly, NR, the NAD^+ precursor, decreases exercise performance in rats [118], most likely due to the pleiotropic metabolic and redox properties of NAD^+ and NADP+. Nicotinic acid also reduced the capacity for high-intensity exercise in humans [119], which is ascribed to lower plasma free fatty acids, leading to earlier fatigue. Studies on NAD^+ precursor supplementation implied prevention of vascular dysfunction, oxidative stress, and muscle age-degeneration in mice [45, 46, 120]. Accordingly, it is important to preserve a high NAD^+ to NAD^+/NADH ratio that can be achieved also by sports activity.

5. Circadian Rhythms, Sleeping Habits, and NAD^+ Levels

Sleep disorders predispose persons to chronic diseases like obesity, depression, diabetes, and many cardiometabolic diseases, which are significantly associated with mortality and morbidity [121, 122] [123–125]. Contrary, a steady pattern of waking and sleeping is associated with health promotion and longevity [126]. Prolonged disruptions of circadian rhythms are associated with negative health consequences [127]. NAD^+ levels and sirtuin activity regulate a healthy circadian rhythm of sleep and wakefulness; concurrently, the NAD^+ level is supervised by circadian rhythm and involved in the circadian clock regulation. NAD^+ levels oscillate with a 24 h rhythm; these can be modified by feeding and sleeping time [128–130]. The central internal clock is in the hypothalamic suprachiasmatic nucleus, and the circadian rhythms are coordinated by intracellular proteins called "circadian clocks." These proteins are regulated by a transcriptional negative feedback loop between transcriptional activators CLOCK and BMAL1 and repressors CRY and PER. CLOCK, the core circadian regulator, is a histone acetyltransferase whose activity is outweighed by the nicotinamide adenine dinucleotide- (NAD^+-) dependent histone deacetylase SIRT1 [131, 132]. CLOCK : BMAL1 heterodimer balances the circadian expression of NAMPT, which regulates the NAD^+ biosynthesis. The activity of NAMPT is constrained by light—or sleep—deprivation and upregulated by darkness and night [130].

With ageing, NAMPT activity declines, and consequently, NAD^+ bioavailability drops [7, 133], leading to the deterioration of the circadian rhythm (change in amplitude, period, and phase). Disrupted circadian rhythms were reported in many pathological conditions including cardiovascular diseases, diabetes, cancer, and accelerated ageing [134, 135]. On the contrary, matching the innate circadian period results in health improvements [135–140].

6. Environmental Stress: Heat/Cold Shock and NAD^+ Levels

Exposure to the elevated heat for short time periods can result in beneficial health effects. Cardiovascular responses to long-term adaptations in response to heat stress result in reduced blood pressure and arterial stiffness and improved endothelial and microvascular function [141]. For example, regular sauna bathing may be linked to several health benefits, which include decreased risk of sudden cardiac death and cardiovascular and all-cause mortality [142], reduction in the risk of neurocognitive diseases and nonvascular conditions such as pulmonary diseases, and amelioration of conditions such as arthritis, headache, and flu [143]. What is more, heat stress cardioprotection and improved postischemic functional recovery in the heat-stressed hearts after cardioplegic arrest due to increased NAD^+ and NADP+ concentrations were observed [144]. Heat shock triggers an increase in the NAD^+/NADH ratio as a result of decreased NADH levels and an increase in recruitment of SIRT1 to the hsp70 promoter [25]. Enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT), which catalyzes nicotinamide adenine dinucleotide (NAD^+) synthesis, is elevated during conditions of heat shock and transcriptionally regulated by the heat shock factor (HSF) and hypoxia-inducible factor 1α (HIF1α) *in vivo* [145, 146].

In addition to heat stress, also cold stress-induced physiological responses and activation of brown adipose tissue (BAT) have health benefits [147]. BAT mainly burns energy in contrast to white adipose tissue (WAT), which stores fat [141]. In mouse and human BAT, cold exposure activates NAD^+ biosynthesis mediated by a rate-limiting enzyme, NAMPT [148]. BAT is abundant in mitochondria and plays a role in energy expenditure related to producing heat by an energy-dissipating process of nonshivering thermogenesis, leading to changes in lipid metabolism [149] and other health benefits like the absence of low-grade inflammation, increased insulin sensitivity, and decreased liver fat [150, 151]. Degradation, whitening, and impaired function of BAT promotes obesity [152–155].

The facts supporting the "NAD^+ > SIRTs > positive effect" pathway as the mechanism of action for the beneficial effects of NAD^+ repletion strategies have been presented so far. Are there indications of concerns about increasing the levels of NAD^+?

7. Potential Deleterious Effects of Increased NAD^+

As already discussed, NAD^+ precursors, nicotinic acid (NA), and NR decreased exercise performance in young rats [118] and reduce the capacity for high-intensity exercise in humans [119], although old individuals seem to benefit from NR supplementation. Namely, increased NAD(P)H levels, decreased oxidative stress, and improved physical performance were observed only in the old subjects [156]. Kourtzidis et al. [157] expressed concern that redox agents administered exogenously in healthy young populations (not suffering from antioxidant deficiency) might lead to adverse effects. Nicotinamide (NAM) overdose was reported to cause hepatotoxicity in rare cases [158]. In addition, it was observed that a high dose of dietary NR caused glucose intolerance and dysfunction of the white adipose tissue in mice fed a slightly obesogenic diet [159].

Regarding longevity, overexpression of SIRT1 was found not to extend lifespan in mice fed standard diets, although they had better general health and fewer carcinomas [160]. Mitchell et al. [43] observed that supplementation with NAM in the mouse model did not change the lifespan, in spite of the improved healthspan. Additionally, Chen et al. [161] challenge the paradigm that CR induces SIRT1 activity in all tissues. Similarly, Frederick et al. [162] suggest that NMN and NR increase in NAD biosynthesis is cell- or tissue-specific.

It appears that the NAD^+ levels could have both procancer and anticancer effects, as NAD^+ is a critical protective factor in early cancer development and could become a damaging factor later in the phase of cancer progression and promotion. Namely, during cancer promotion, progression, and treatment, increased NAD^+ levels could have adverse effects on the malignancy process due to increased cell survival, growth advantage, increased resistance to radio- and chemotherapy, and promotion of inflammation. In contrast, NAD^+ restoration could prevent or reverse the phenotype of malignant cells in the early stages by inducing cellular repair and adaptive stress responses and regulating cell cycle arrest and apoptotic removal of damaged cells (reviewed in [24]). In addition, the compound FK866, which inhibits the nicotinamide recycling enzyme NAMPT, is a tumor apoptosis inducer due to the NAD^+ depletion [163, 164] and is used as an anticancer drug.

In the area of inflammation/sepsis, there is also controversy regarding the NAD(+)-dependent sirtuin family, as elevated NAD(+) levels play a different role in the different stages of sepsis. In the initial (proinflammatory) phase, which is characterized by a cytokine storm, overproduction of reactive oxygen species (ROS), and metabolic shift [165], SIRT1 activation shows positive effects, whereas the SIRT1 expression should be inhibited in the later stages of sepsis [166]. Therefore, due to the dynamic phases of sepsis, the role of SIRT1 cannot simply be defined as beneficial or detrimental. Increased NAD^+ might have also negative effects on inflammatory disorders, such as rheumatoid arthritis due to stimulated inflammatory cytokine secretion by leukocytes [167].

Another potential risk could be posed by the toxic degradation products and metabolites of NAD^+ precursors, e.g., nicotinic acid adenine dinucleotide (NAAD), *N*-methyl nicotinamide (MeNAM), and 2-PY[71, 168]. Lastly, increased NAM levels due to the supplementation with NAD^+ precursors (NAM, NR, or NMN) could inhibit PARPs and CD38 activities [169], while SIRT1 feedback inhibition *in vivo* by NAM may not be so important [170, 171]. Increased levels of NAM might alter also the methyl pool used to methylate DNA and proteins [171].

8. Conclusions

It is not only the NAD/NADH redox role as hydride and electron transfer in redox metabolic reactions but mainly the NAD^+ as the signalling molecule and substrate for sirtuins and PARPs that is responsible for the health benefits and longevity. Cellular NAD^+ content and an adequate NAD^+/NADH ratio can postpone pathologic processes associated with impaired cell signalling and mitochondrial function [87, 172, 173]. Thus, for maintaining optimal cellular functioning and organismal health, it is necessary to implement the lifestyle approaches that stimulate increased NAD^+ levels. The synergistic effects of different measures to ensure a healthy lifestyle are important, as there is an intimate and reciprocal relationship between them. For example, sedentary lifestyle, overeating, and excessive intake of fat and sugar are associated with disturbances in circadian rhythms [174, 175] and downregulation of NAMPT gene expression [4]. Implementation of the time-restricted feeding without reducing the caloric intake (8 h per day feeding/16 h per day fasting) improved the robustness of circadian and metabolic rhythms and prevented metabolic diseases in mice on a high-fat diet [176]. Lifestyle approaches, such as exercise and CR, can reverse insulin resistance and type 2 diabetes mellitus (T2DM) [12]. Both manipulations increase the NAMPT-mediated NAD^+ generation, activate mechanistic pathways of AMPK, and enhance the SIRT1 activity and mitochondrial function [4, 114, 177, 178]. Sirtuins affect various cellular processes, including lipid metabolism, insulin

secretion, and sensitivity [179]. NAD^+ levels within cells are regulated by its precursors' intake, biosynthetic pathways, and degradative enzymes [180], which can be additionally balanced by selected lifestyle factors discussed here. In order to provide sufficient NAD^+ bioavailability and appropriate expression of NAMPT, it is necessary to ingest sufficient amounts of NAD^+ precursors/intermediates in the vitamin B3 forms, preferably as a part of a normal diet, to practice regular and moderate sports activity, and to observe time intervals between darkness and light exposure as well as the appropriate time intervals between feeding and fasting.

The presented studies support the hypothesis that maintaining NAD^+ levels leads to healthy cell metabolism, which is beneficial in terms of amelioration of metabolic diseases and ageing. It should be stressed that NAD^+ is not the only factor, but rather one of the several components that influence cell health. There are many other positive effects of calorie restriction, eating habits, exercise, circadian rhythms, and environmental stress on human health that are beyond the scope of this paper. Although many animal studies have shown the link between NAD^+ and healthspan, the complex role of NAD^+ in the etiology of ageing and age-related chronic diseases in humans should be further elucidated. The current state of knowledge about NAD^+ positive effects on ageing and healthspan is mainly based on experiments on cell cultures and model organisms, so that the positive health effects of NAD^+ in humans will need to be confirmed in future in-depth studies and clinical trials.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Moderate Mechanical Stimulation Protects Rats against Osteoarthritis through the Regulation of TRAIL via the NF-κB/NLRP3 Pathway

Yue Yang,[1] Yang Wang,[2] Yawei Kong,[3] Xiaoning Zhang,[1] He Zhang,[1] Xinyuan Feng,[1] Ziyuan Wang,[1] Peng Gao,[1] Mingyue Yan,[1] Lunhao Bai,[1] and Feng Li[4]

[1]*Department of Orthopedic Surgery, Shengjing Hospital of China Medical University, Shenyang 110000, China*
[2]*Department of Ultrasound, Shengjing Hospital of China Medical University, Shenyang 110000, China*
[3]*International Patient Center, Brigham and Women's Hospital, Harvard Medical School, Boston 02115, USA*
[4]*Department of Cell Biology, China Medical University, Shenyang 110000, China*

Correspondence should be addressed to Lunhao Bai; bailunhao@126.com

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The aim of this study was to examine exercise-related genes in articular cartilage identified through bioinformatics analysis to dissect the potential signaling pathway involved in mechanical stimulation in osteoarthritis (OA). To this end, we evaluated the GSE74898 dataset from the Gene Expression Omnibus database for exercise-related differentially expressed miRNAs (DE-miRNAs) using the R software package and predicted potential target genes for these miRNAs using miRTarBase. Functional annotation and pathway enrichment analysis were performed for these potential DE-miRNA targets. The effects of mechanical stimulation on the tumor necrosis factor-related apoptosis-induced ligand (TRAIL)/nuclear factor-kappa B (NF-κB)/nucleotide-binding and oligomerization domain-like receptor containing protein 3 (NLRP3) signaling pathway were evaluated in articular cartilage and chondrocytes. A total of 394 DE-miRNAs were identified (103 upregulated miRNAs; 291 downregulated miRNAs) in the cartilage of rats following treadmill exercise compared to the cartilage of unexercised control rats. Thus, mechanical stimulation could modulate the TRAIL/NF-κB/NLRP3 signaling pathway on OA. Histological and protein analysis demonstrated that moderate-intensity treadmill exercise could ameliorate OA through the downregulation of TRAIL. Furthermore, moderate cyclic tensile strain (CTS) could rescue chondrocytes from the effects of TRAIL via the inhibition of the nuclear translocation of NF-κB p65 and formation of NLRP3. Our findings indicate that moderate mechanical stimulation could ameliorate the degeneration of cartilage and chondrocyte damage through the inhibition of the TRAIL/NF-κB/NLRP3 pathway.

1. Introduction

Osteoarthritis (OA) is a whole joint disease that involves structural alterations in the articular cartilage, subchondral bone, ligaments, capsule, synovium, and periarticular muscles [1]. It affects a large portion of the population worldwide [2, 3]. Mechanical loading-based interventions are critical elements in OA treatment plans [1, 4–6].

Chondrocytes play a central role in maintaining cartilage homeostasis [7, 8]. They are mechanically sensitive and can adapt to the level of mechanical stimuli [9]. Chondrocyte mechanotransduction signaling pathways are complex, and the mechanisms by which chondrocytes convert mechanical stimuli into biochemical signals need further investigation.

MicroRNAs (miRNAs) play a role in the regulation of many biological processes [10–12]. Many miRNAs contribute to the chondrocyte phenotype via signaling pathways [13, 14]. Based on miRTarBase, gene ontology (GO) annotation, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (GSE74898), we predicted that the signaling pathway involving tumor necrosis factor-related apoptosis-induced ligand (TRAIL), nuclear factor-kappa B (NF-κB), and nucleotide-binding and oligomerization domain-like receptor containing protein 3

(NLRP3) played a key role in the mechanism by which mechanical stimulation ameliorates OA.

In the present study, we evaluated TRAIL expression levels in OA articular cartilage in rats following treadmill exercise compared to unexercised control animals. We also investigated changes in the NF-κB/NLRP3 signaling pathway following the treatment of chondrocytes with mechanical stress in combination with TRAIL as a potential mechanism for the chondroprotective effect of moderate mechanical stimulation in OA.

2. Materials and Methods

2.1. miRNA Microarray. In the discovery step of this study, we used datasets that compared the miRNA expression of exercise-related genes in cartilage. The titles and abstracts of these datasets were screened, and those of interest were further investigated. Based on this analysis, the GSE74898 dataset was selected for further study. This dataset, which is based on the GPL6247 platform (Affymetrix Rat Gene 1.0 ST Array), contained three healthy Sprague-Dawley (SD) rats without exercise and 12 healthy Sprague-Dawley (SD) with exercise for a different number of days (2, 5, and 15 days) and was downloaded from the National Center for Biotechnology Information (NCBI) GEO database (https://www.ncbi.nlm.nih.gov/geo).

2.2. Prediction of Target Genes and miRNA-Gene Network Construction. The potential target genes of the identified DE-miRNAs were predicted using miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php), which is an experimentally validated micro-RNA-target interaction database [12]. The target genes were mapped in the STRING database (http://string-db.org) to assess the functional associations among these target genes [15]. Only interactions with a combined score of greater than 0.4 were considered as significant.

2.3. GO and Pathway Analysis. The database for annotation, visualization, and integrated discovery (DAVID 6.8, http://david-d.ncifcrf.gov/) was used to perform functional annotation and pathway enrichment analysis (GO and KEGG pathway analysis [16]) for the predicted targets of the selected DE-miRNAs. A p value of less than 0.05 was considered statistically significant.

2.4. Experimental Animals. Fifty male Sprague-Dawley (SD) rats (230 ± 10 g; specific-pathogen-free) were obtained from HFK Bioscience Co. Ltd. (Beijing, China). This study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (no. 2017PS237K). The maintenance and care of the experimental rats followed the guidelines of the committee as previously described [17–19].

2.5. OA Model and Treadmill Running Protocols. The OA model and adaptive treadmill exercise protocols used in this study were based on previous studies [17–19]. After the adaptive treadmill exercise, the rats were randomly divided into five study groups ($n = 10$ in each group): control group (CG): intra-articular injection of 50 μl sterile saline per cavity; OAG1, OAG2, and OAG3: intra-articular injection of 0.2 mg, 0.5 mg, or 1.0 mg MIA, respectively, per cavity in 50 μl sterile saline; and OAE: OAG3 conditions with moderate treadmill exercise. The rats in groups CG, OAG1, OAG2, and OAG3 were kept sedentary, whereas the rats in group OAE began the treadmill exercise program 24 h after injection of MIA. These rats exercised at a speed of 12 m/min for 45 min once daily for five days per week for four weeks with appropriate photostimulation, acoustic stimulation, and electric stimulation (Figure 1).

2.6. Sampling and Tissue Preparation. After the last formal treadmill exercise session, the animals were anesthetized with 1.5% pentobarbital sodium 0.2 ml/100 g, intraperitoneal injection. The collection of serum, intra-articular lavage fluid (IALF), and articular cartilage was performed as previously described [17–19].

2.7. Histology. The method for hematoxylin and eosin and toluidine blue staining was performed as previously described [17–19]. Injury to the articular cartilage in the femur and tibia was assessed independently using the modified Mankin score [20] with a scale of 0 to 14 and the Osteoarthritis Research Society International (OARSI) score with a scale of 0 to 24 [21]. The tibial and femoral cartilage scores were then added, such that the maximum possible Mankin score was 28 and the maximum OARSI score was 48. Two experienced observers (Yue Yang and Xiaoning Zhang) performed the scoring in a blinded manner.

2.8. Immunohistochemistry. Immunohistochemistry was performed as previously described [17–19]. Collagen II levels are presented relative to CG. MMP-13 and NF-κB p65 were expressed as the percentage of positive cells.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). TNF-α and IL-1β levels in the knee IALF and serum were determined using ELISA kits (Tongwei, Shanghai, China) according to the manufacturer's instructions. The protein content in the IALF was measured to ensure that the ratio of dilution was equal.

2.10. Western Blotting. The primary antibodies are as follows: rabbit polyclonal anti-collagen II antibody (ab34712, 1 : 5,000; Abcam), molecular weight 142 kDa; rabbit monoclonal anti-NLRP3 antibody (ab210491, 1 : 1,000; Abcam), molecular weight 118 kDa; rabbit polyclonal anti-NF-κB p65 antibody (AB21014, 1 : 500; AbSci), molecular weight 65 kDa; rabbit monoclonal anti-caspase-1 antibody (ab108362, 1 : 5,000; Abcam), molecular weight 45 kDa; rabbit monoclonal anti-pro caspase-1 antibody (ab179515, 1 : 1,000; Abcam), molecular weights 45 kDa and 42 kDa; mouse monoclonal anti-β-actin (60008-1-lg, 1 : 5,000, Proteintech Group), molecular weight 42 kDa; rabbit monoclonal anti-IκB-α antibody (ab32518, 1 : 10,000; Abcam), molecular weight 36 kDa; rabbit polyclonal anti-TRAIL antibody (ab231063, 2 μg/ml; Abcam), molecular weight 33 kDa; rabbit polyclonal anti-IL-1β antibody (ab150777, 1 : 1000; Abcam), molecular weight 31 kDa; and rabbit polyclonal anti-histone H2A.X (AB41012, 1 : 1,000, AbSci), molecular

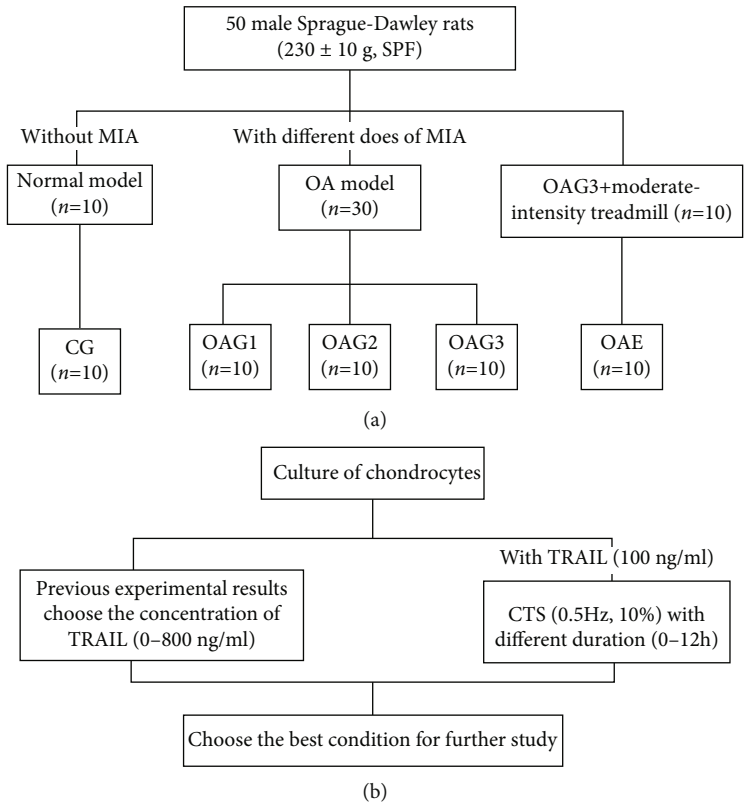

Figure 1: The design of the treatment schedule. (a) The design of the animal experiment. Experimental groups: CG: control group; OAG1, OAG2, and OAG3: OA groups injected with different doses of MIA; OAE: OAG3 subjected to moderate-intensity treadmill exercise. (b) The design of the chondrocyte experiment. CTS: cyclic tensile strain; TRAIL: tumor necrosis factor-related apoptosis-induced ligand; MIA: monoiodoacetate; OA: osteoarthritis; SPF: specific-pathogen-free.

weight 19 kDa. β-Actin and histone H2A.X were used as internal controls. The processes of western blotting are consistent with previous articles [17–19].

2.11. Isolation and Culture of Chondrocytes. The isolation and culture of chondrocytes were performed as previously described [17].

2.12. Cell Counting Kit-8 (CCK-8) Assay. Primary rat chondrocytes were seeded in 96-well plates (5×10^3 cells per well) and cultured for one day at 37°C in DMEM culture medium containing 10% FBS until 70 to 80% confluency. The medium was changed to serum-free DMEM containing increasing concentrations of TRAIL (abs01000, 0, 25, 50, 100, 200, 400, and 800 ng/ml), and cells were cultured for 12 h at 37°C. After TRAIL treatment, 10 μl CCK-8 (Beyotime, C0042) and 90 μl serum-free DMEM were added to each well followed by incubation for 2 h at 37°C. The absorbance at 450 nm was measured using a spectrophotometer (Synergy H1, BioTek, USA).

2.13. Exposure of Chondrocytes to CTS. Chondrocytes were grown on collagen I-coated BioFlex 6-well culture plates (Flexcell International, Hillsborough, NC) to 80% to 90% confluency. CTS experiments were performed using the FX-5000 Flexcell system (Flexcell International, McKeesport, PA). The plates were placed on a loading station in an incubator with 5% CO_2 such that when the vacuum was applied to the loading station, the membrane deformed across the post face, creating uniform biaxial strain. Chondrocytes were subjected to CTS (10%, 0.5 Hz) for 0, 1, 2, 4, 8, or 12 h in the presence of 100 ng/ml TRAIL. The stimulation of CTS and TRAIL on chondrocytes began at the same time. A 4 h treatment with CTS was determined to be the optimal duration and was used for subsequent experiments. All chondrocytes were harvested at 12 h with TRAIL.

2.14. Quantitative Real-Time Polymerase Chain Reaction (qPCR). The processes of qPCR are consistent with previous articles [19]. Expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [22] using β-actin as the reference gene. The primer pair sequences were specific to rat MMP-13 (F-5′-TGATGATGAAACCTGGACAAGCA-3′; R-5′-GAACGTCATCTCTGGGAGCA-3′), MMP-1 (F-5′-TGTTCGCCTTCTACAGAGGAGACC-3′; R-5′-TGTCGGTCCACGTCTCATCCAG-3′), and β-actin (F-5′-GGAGATTACTGCCCTGGCTCCTA-3′; R-5′-GACTCATCGTACTC CTGCTTGCTG-3′).

2.15. Cellular ROS Production. Chondrocytes were seeded in 6-well plates (1.5×10^6 cells/well). Chondrocytes were exposed to different doses of TRAIL (abs01000, 25, 50, or 100 ng/ml) for 12 h with or without CTS (10%, 0.5 Hz, 4 h). All chondrocytes were harvested at 12 h with TRAIL

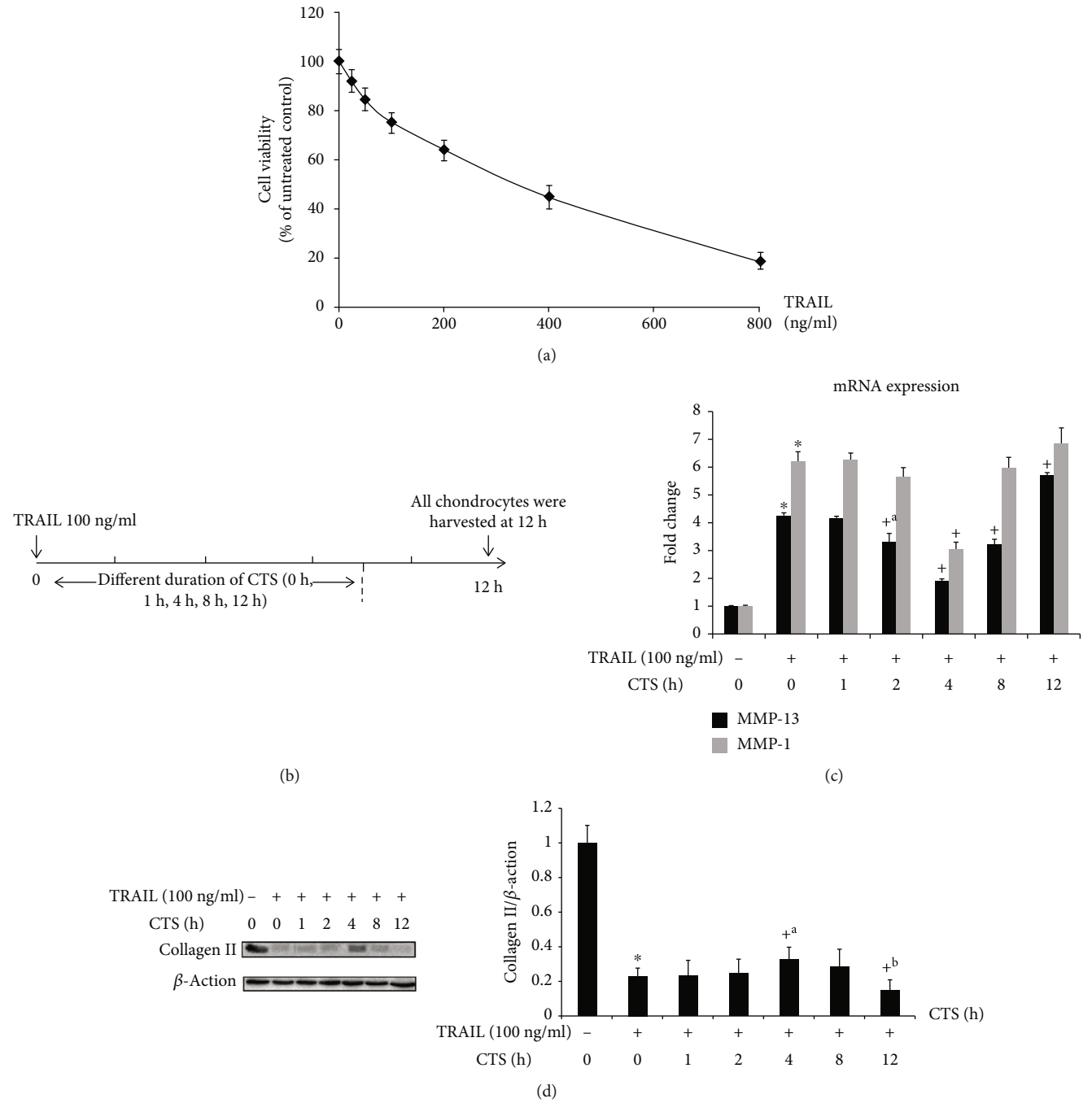

Figure 2: Western blot and qPCR analysis of chondrocytes treated with CTS for different durations. (a) The results of CCK-8 test. (b) The design of chondrocytes was subjected to CTS (10%, 0.5 Hz) for different durations (0 h, 1 h, 2 h, 4 h, 8 h, and 12 h) with TRAIL. (c) MMP-13 and MMP-1 mRNA expression in chondrocytes following different durations of CTS (10%, 0.5 Hz) was determined by qPCR. Differences between untreated and TRAIL (100 ng/ml)-induced chondrocytes ($^{*}p < 0.001$) and TRAIL-induced chondrocytes and those subjected to CTS for different durations ($^{+}p < 0.001$, $^{+a}p = 0.001$) were significant (ANOVA). Data are presented as the mean ± 95%confidence intervals; $n = 9$ per group. (d) Collagen II protein levels in chondrocytes. Differences between untreated and TRAIL (100 ng/ml)-induced chondrocytes ($^{*}p < 0.001$) and TRAIL-induced chondrocytes and those subjected to CTS of different durations ($^{+}p < 0.001$, $^{+a}p = 0.006$, and $^{+b}p = 0.002$) were significant (ANOVA). β-Actin was used as an internal control. Data are presented as the mean ± 95%confidence intervals ; $n = 3$ per group.

(Figure 2(b)). ROS production was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (S0033, Beyotime),s which is directly oxidized by ROS (e.g., superoxide ion, hydrogen peroxide, and hydroxyl). Chondrocytes were incubated with 10 μM DCFH-DA for 45 min at 37°C in the dark and then washed three times in PBS. Fluorescence was detected by fluorescent microscopy and measured with the BD FACSCalibur (488 nm, excitation; 525 nm, emission).

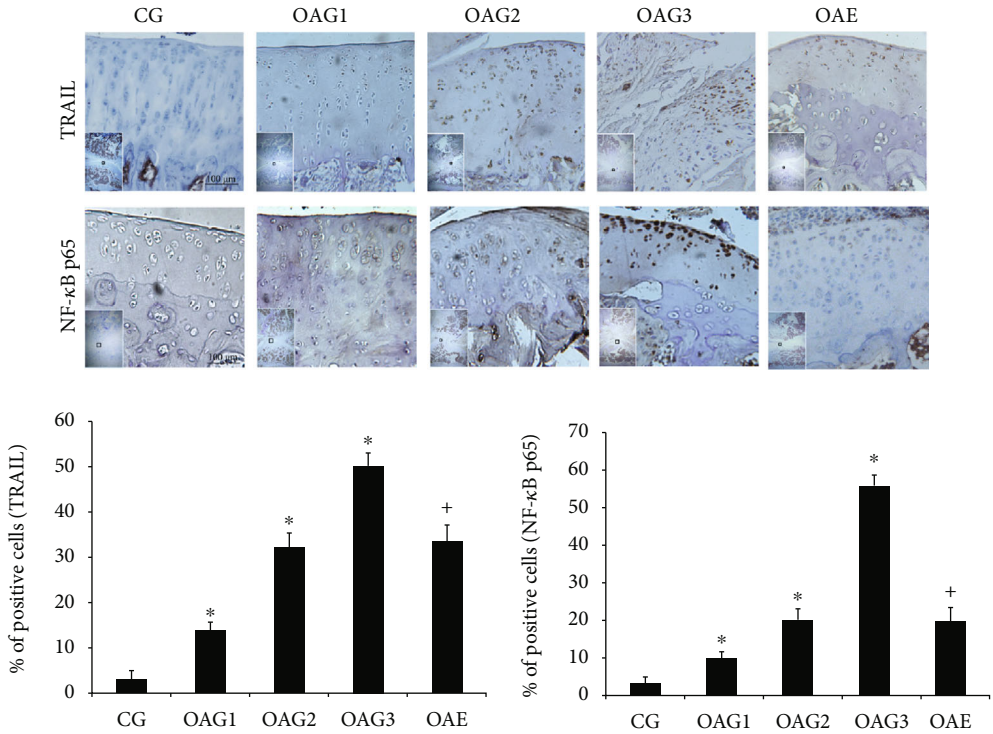

Figure 3: Immunohistochemical staining. The micrographs show the percentages of TRAIL-positive cells and the percentage of NF-κB p65 nuclear translocation in the articular cartilage of each experimental group. Differences between CG and OAG1, OAG2, and OAG3 ($^{*}p < 0.001$) and OAG3 and OAE ($^{+}p < 0.001$) were significant (ANOVA). Data are presented as the mean ± 95%confidence intervals; $n = 5$ rats per group. Experimental groups: CG: control group; OAG1, OAG2, and OAG3: OA groups treated with different doses of MIA; OAE: OAG3 subjected to moderate-intensity treadmill exercise.

2.16. Detection of Apoptosis by Flow Cytometry. Chondrocytes were washed three times with PBS, resuspended in 500 μl binding buffer, and collected as described above. The cells were double-stained using the Annexin V-FITC/propidium iodide (PI) staining kit (AD101; Dojindo, Japan), which is a standard method to distinguish between cells dying via apoptosis or necrosis. The total percentage of the apoptotic cells is the sum of both the early and late stages of apoptosis (Annexin V-FITC positive). The cells that stained only with PI were considered the necrotic cell population. Data acquisition and analysis were performed using CellQuest software (BD Biosciences, San Jose, CA, USA).

2.17. Immunofluorescence Analysis of Chondrocytes. Immunofluorescence was performed as previously described [17].

2.18. Statistical Analysis. Data are expressed as the means with 95% confidence intervals (CIs) and analyzed using SPSS statistical software version 22 (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk and Levene's tests were applied to evaluate the normality and homogeneity of the results, respectively. For variables that exhibited normal distribution, data were analyzed using one-way analysis of variance (ANOVA). A p value of less than 0.05 indicated statistical significance.

3. Results

3.1. Identification of DE-miRNAs and Their Target Genes. Analysis of the GSE74898 miRNA array identified a total of 565, 430, and 618 miRNAs that were differentially expressed more than twofold in the cartilage of unexercised rats compared to that of rats exercised for 2, 5, or 15 days, respectively. A total of 394 DE-miRNAs were common across the three time points, including 103 upregulated and 291 downregulated miRNAs. The volcano plot and Venn diagram for these DE-miRNAs are presented in Fig. S1.

3.2. GO Functional Enrichment Analysis. Four categories of GO functional annotation analysis were performed on the potential target genes, including molecular functions, biological processes, cellular components, and biological pathways. As shown in Fig. S2, the most enriched GO functions for these DE-miRNA target genes included motor activity, signal transduction, extracellular, and the TRAIL signaling pathway in these four categories, respectively.

3.3. KEGG Pathway Enrichment Analysis. KEGG pathway enrichment analysis was conducted to further analyze the enriched pathways for these target genes. The enriched KEGG pathways included the B cell receptor signaling pathway, cell cycle, Fc gamma R-mediated phagocytosis, systemic lupus erythematosus, Fc epsilon RI signaling pathway, oocyte meiosis, NF-kappa B signaling pathway,

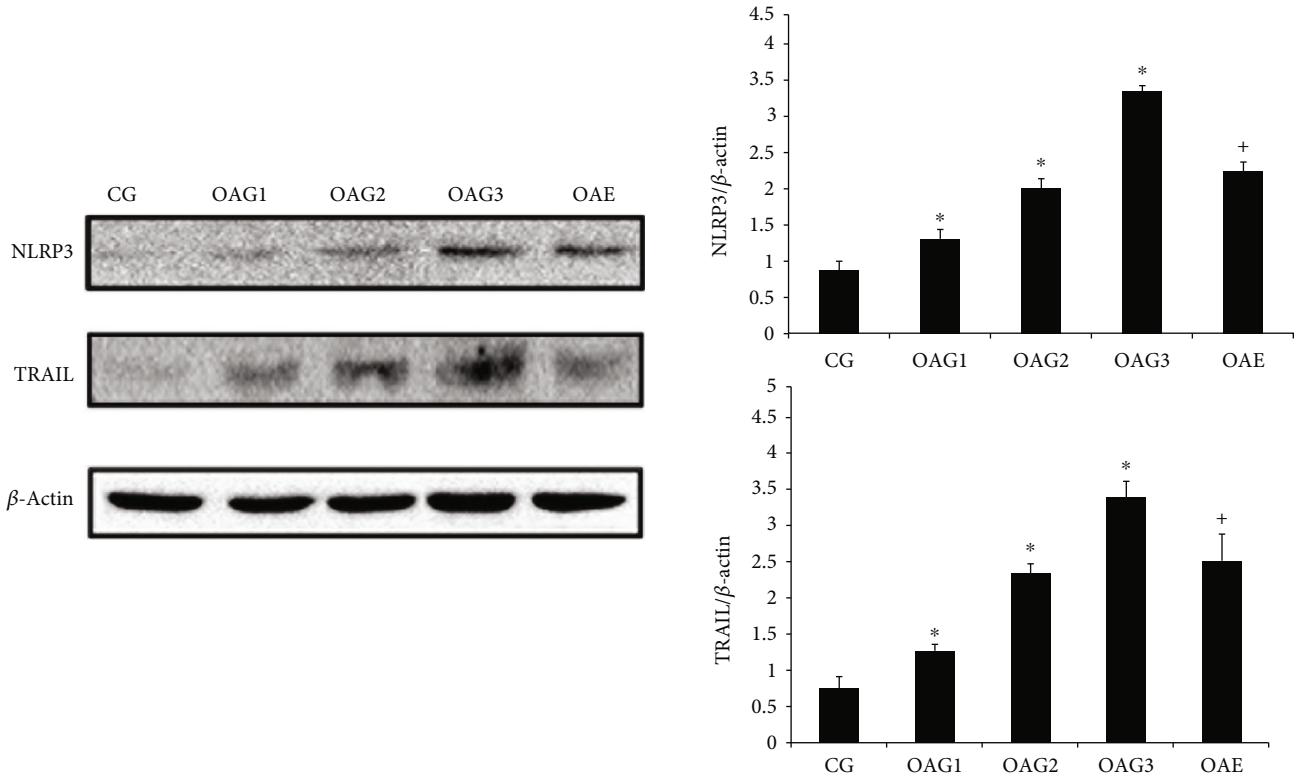

Figure 4: Protein expression levels were determined by western blotting of total protein extracted from cartilage. Differences between CG and OAG1, OAG2, and OAG3 ($^{*}p < 0.001$) and OAG3 and OAE ($^{+}p < 0.001$) were significant (ANOVA). β-Actin was used as internal controls. Data are presented as the mean ± 95%confidence intervals; $n = 3$ rats per group. Experimental groups: CG: control group; OAG1, OAG2, and OAG3: OA groups treated with different doses of MIA; OAE: OAG3 subjected to moderate-intensity treadmill exercise.

viral carcinogenesis, p53 signaling pathway, and chemokine signaling pathway (Fig. S3B). We predicted the involvement of the TRAIL signaling pathway in the exercise-induced changes in cartilage through the GO functional enrichment analysis. Because TRAIL can activate the NF-κB signaling pathway, it could be the link between mechanical stimulation and the responses of chondrocytes to proinflammatory cytokines [8]. Thus, the TRAIL/NF-κB signaling pathway may play a role in the effects of mechanical stimulation on OA.

3.4. Histological and Immunohistochemical Analysis. The results of histological assessment (Mankin and OARSI scores) and collagen II are consistent with previous results with different concentrations [17] (data not shown).

Immunohistochemical staining (TRAIL and NF-κB p65) has similar results with Mankin and OARSI scores (the percentages of TRAIL-positive cells: CG = 3.2, 95% CI 1.4-4.9; OAG1 = 13.9, 95% CI 12.2-15.6; OAG2 = 32.3, 95% CI 29.3-35.3; OAG3 = 50.3, 95% CI 47.4-53.1; and OAE = 33.4, 95% CI 29.9-36.9; the percentage of NF-κB p65 nuclear translocation: CG = 3.1, 95% CI 1.4-4.9; OAG1 = 9.9, 95% CI 8.3-11.5; OAG2 = 20.0, 95% CI 18.0-22.0; OAG3 = 55.9, 95% CI 50.7-61.1; and OAE = 19.7, 95% CI 16.4-23.0) (Figure 3).

3.5. TNF-α and IL-1β ELISA. The results of TNF-α and IL-1β are consistent with previous results with different concentrations [17] (data not shown).

3.6. Western Blot Analysis. The results of collagen II and NF-κB p65 in the cartilage are consistent with previous results with different concentrations [17] (data not shown).

The changes in the levels of NLRP3 and TRAIL in the cartilage between the different groups paralleled those observed by histology and immunohistochemistry (Figure 4).

TRAIL induced a significant decrease in collagen II compared to untreated chondrocytes, but the collagen II levels increased in the chondrocytes following a 4 h treatment with CTS (Figure 2(d)). TRAIL caused a dose-dependent decrease in collagen II and IκB-α protein levels and a dose-dependent increase in NLRP3, procaspase-1, IL-1β, and caspase-1 protein levels in chondrocytes. However, CTS treatment for 4 h ameliorated these changes (Figure 5(a)).

3.7. qPCR Assay. The relative expression levels of MMP-13 and MMP-1 in chondrocytes are shown in Figure 2(c). TRAIL caused an increase in the expression of both MMP-13 and MMP-1; however, these increased levels were decreased by 4 h treatment with CTS (relative expression levels of MMP-1: control = 1.00, 95% CI 0.97-1.03; TRAIL only = 6.19, 95% CI 5.83-6.54; TRAIL+CTS 1 h = 6.25, 95% CI 5.99-6.51; TRAIL+CTS 2 h = 5.65, 95% CI 5.32-5.97; TRAIL+CTS 4 h = 3.05, 95% CI 2.82-3.28; TRAIL+CTS 8 h = 5.95, 95% CI 5.53-6.37; and TRAIL+CTS 12 h = 6.81, 95% CI 6.22-7.41; relative expression levels of MMP-13: control = 1.00, 95% CI 0.99-1.01; TRAIL only = 4.34, 95%

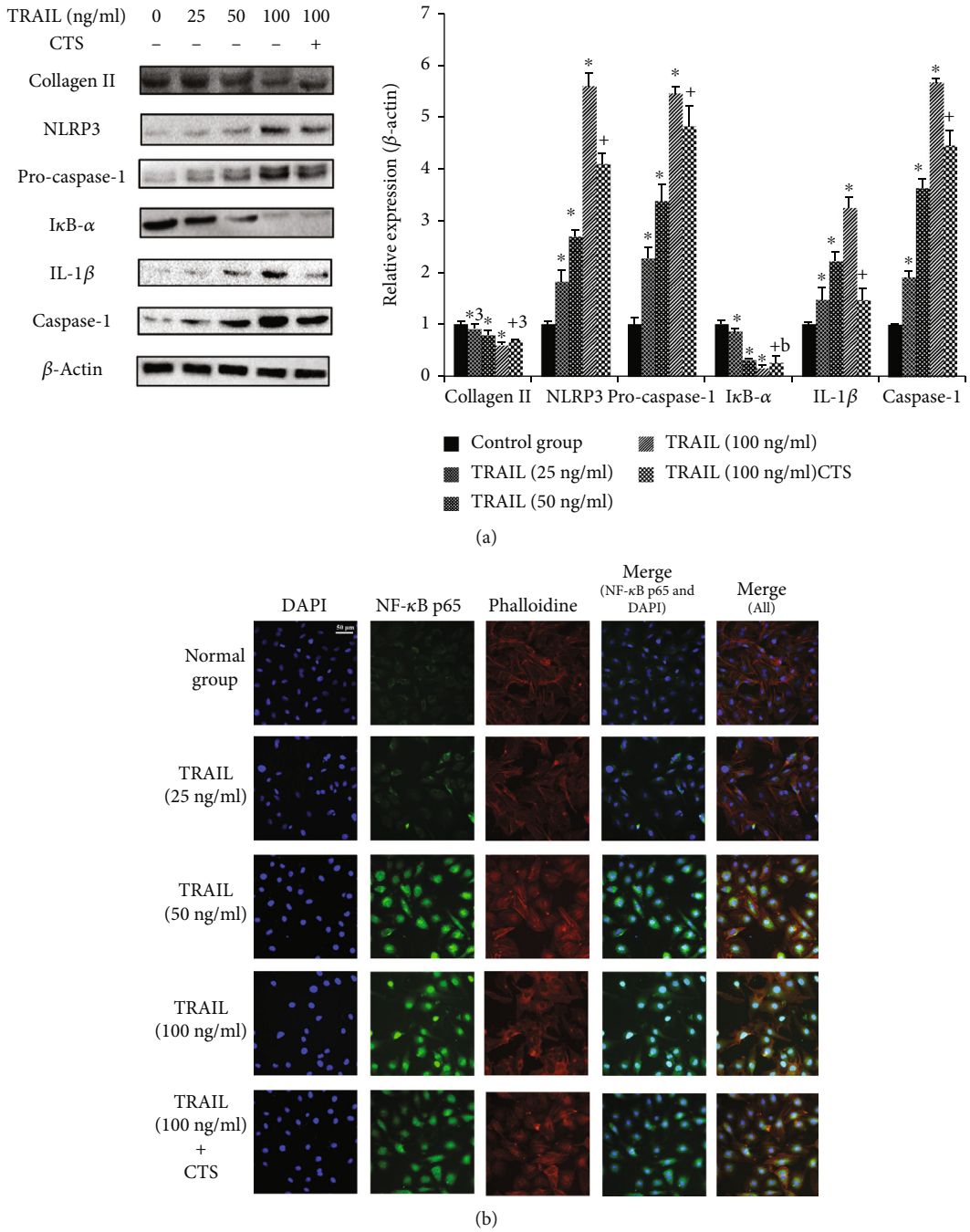

Figure 5: Western blot and immunofluorescence analysis of chondrocytes. (a) Western blotting results for collagen II, NLRP3, procaspase-1, IκB-α, IL-1β, and caspase-1. Differences between untreated and TRAIL (0, 25, 50, and 100 ng/ml)-induced chondrocytes ($^{*}p < 0.001$, $^{*a}p = 0.005$) and TRAIL (100 ng/ml)-induced chondrocytes and those subjected to CTS for 4 h ($^{+}p < 0.001$, $^{+a}p = 0.003$, and $^{+b}p = 0.001$) were significant (ANOVA). Data are presented as the mean ± 95%confidence intervals; $n = 3$ per group. (b) Effects of CTS for 4 h on the nuclear translocation of NF-κB p65 in TRAIL-induced chondrocytes. The chondrocytes were immunostained using anti-NF-κB p65 rabbit antibody (green) and visualized by confocal microcopy. The cytoskeleton was visualized with phalloidin (red), and the cell nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 50 μm.

CI 4.12-4.36; TRAIL+CTS 1 h = 4.16, 95% CI 4.08-4.24; TRAIL+CTS 2 h = 3.30, 95% CI 2.99-3.61; TRAIL+CTS 4 h = 1.89, 95% CI 1.79-1.98; TRAIL+CTS 8 h = 3.25, 95% CI 3.12-3.38; and TRAIL+CTS 12 h = 5.71, 95% CI 5.60-5.81). These data were consistent with the collagen II results.

3.8. Immunofluorescence in Chondrocytes. As shown in Figure 5(b), TRAIL caused significant accumulation of NF-κB p65 protein in the nuclei of chondrocytes compared to the control group. CTS suppressed the nuclear translocation of NF-κB p65 induced by TRAIL (Figure 5(b)).

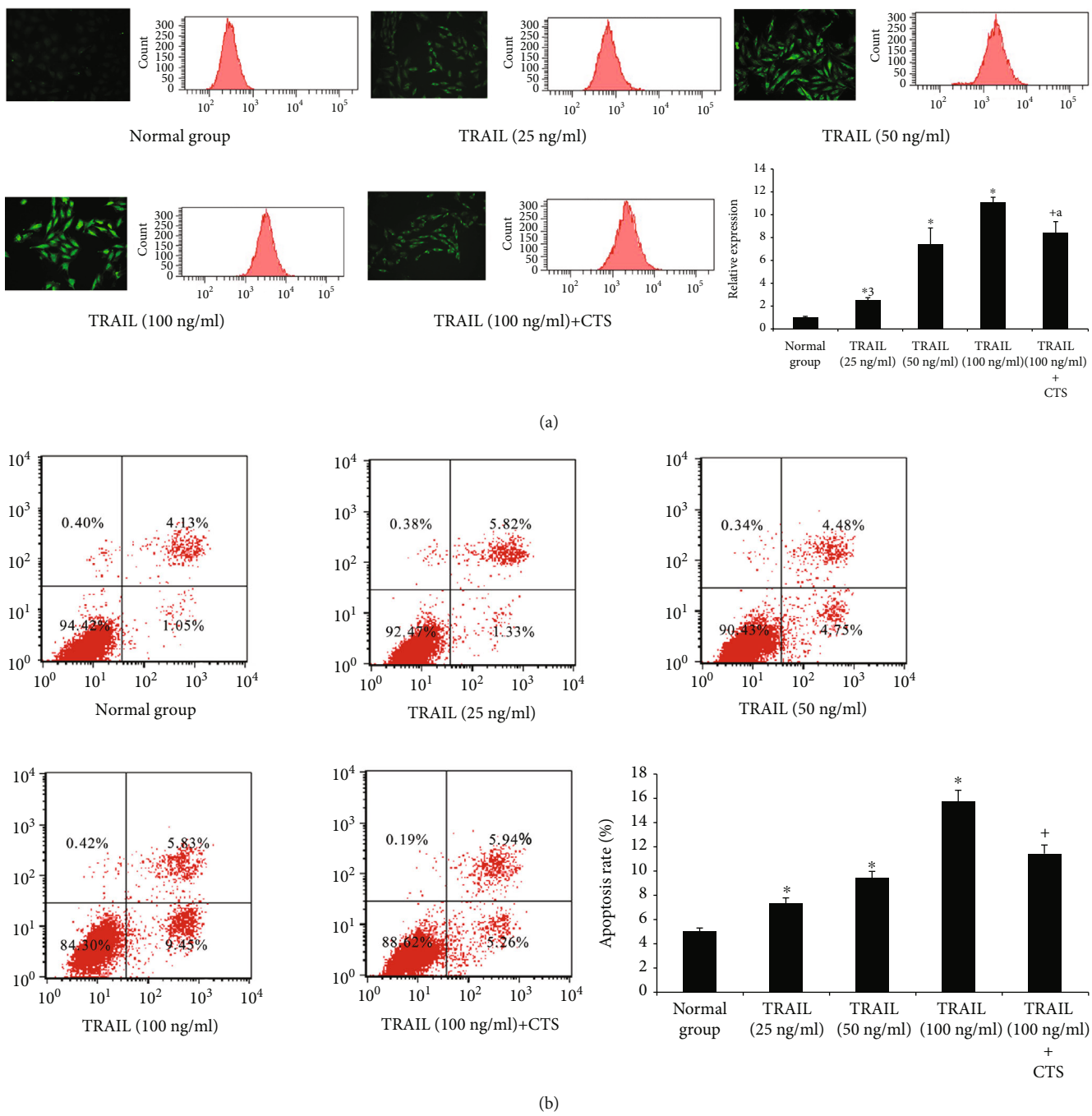

Figure 6: Reactive oxygen species (ROS) and apoptosis in chondrocytes. (a) Fluorescence microscopy and flow cytometry of ROS in chondrocytes. Differences between untreated and TRAIL (0, 25, 50, and 100 ng/ml)-induced chondrocytes ($^{*}p < 0.001$, $^{*a}p = 0.012$) and TRAIL (100 ng/ml)-induced chondrocytes and those subjected to CTS for 4 h ($^{+a}p = 0.012$) were significant (ANOVA). Data are presented as the mean ± 95%confidence intervals; $n = 3$ per group. (b) Chondrocyte apoptosis analysis by flow cytometry. Differences between untreated and TRAIL (0, 25, 50, and 100 ng/ml)-induced chondrocytes ($^{*}p < 0.001$) and TRAIL (100 ng/ml)-induced chondrocytes and those subjected to CTS for 4 h ($^{+}p < 0.001$) were significant (ANOVA). Data are presented as the mean ± 95%confidence intervals; $n = 3$ in each group.

3.9. ROS Analysis of Chondrocytes. ROS production in chondrocytes was enhanced by TRAIL in a dose-dependent manner (Figure 6(a)). CTS significantly reduced the ROS levels induced by TRAIL (relative intensity of ROS: normal group = 1.00, 95% CI 0.94-1.07; TRAIL (25 ng/ml) = 2.55, 95% CI 2.36-2.74; TRAIL (50 ng/ml) = 7.82, 95% CI 5.41-10.23; TRAIL (100 ng/ml) = 10.64, 95% CI 8.33-12.95; and TRAIL (100 ng/ml)+CTS = 8.50, 95% CI 7.56-9.44).

3.10. Analysis of Chondrocyte Apoptosis. The percentage of apoptotic chondrocytes significantly increased in response to treatment will increase the amounts of TRAIL. These

effects were abrogated by moderate CTS (Figure 6(b)) (the percentage of apoptotic chondrocytes: normal group = 5.04, 95% CI 4.68-5.40; TRAIL (25 ng/ml) = 7.37, 95% CI 6.84-7.91; TRAIL (50 ng/ml) = 9.46, 95% CI 8.92-9.99; TRAIL (100 ng/ml) = 15.70, 95% CI 14.74-16.66; and TRAIL (100 ng/ml) + CTS = 11.49, 95% CI 10.86-12.12).

4. Discussion

Exercise is one of the most widely accepted nonpharmacological therapies for OA [23]. However, how exercise affects the pathology of OA is still unclear. As mechanosensitive cells, chondrocytes synthesize the extracellular matrix and depend on intracellular signals generated in response to biomechanical stress [24, 25]. Therefore, it is important to understand the mechanisms underlying the mechanical stimulation of cartilage and chondrocytes. miRNAs are a group of small, endogenous noncoding RNAs that possess a variety of biological functions. Recent studies showed that miRNAs are involved in the regulation of gene expression in cartilage during exercise [15, 16]. In this study, we identified exercise-related miRNAs in rat cartilage through the analysis of GEO datasets. GO and KEGG pathway analysis revealed that the TRAIL/NF-κB/NLRP3 signaling pathway may be of importance.

There were several principal findings in this study. First, there were significant differences observed in the articular cartilage of the knees of rats injected with MIA to induce OA compared to control rats. Exercise had significant therapeutic effects against the MIA-induced OA. Second, the TRAIL levels in the chondrocytes were substantially increased by MIA in a dose-dependent manner. Moderate CTS reduced the inflammatory response induced by TRAIL in the chondrocytes. Third, the suppression of the TRAIL-induced inflammatory response by moderate CTS appeared to occur through the NF-κB/NLRP3 pathway.

To study the connection between mechanical stress and OA progression, we investigated the effect of exercise on an OA rat model induced by the intra-articular injection of MIA. Our results indicate that there were significant differences in both the histology (Mankin and OARSI scores) and collagen II, NLRP3, TRAIL, and NF-κB p65 protein levels between the articular cartilage of the knees of MIA-treated and control rats. Synovitis plays a key role in the pathogenesis of OA and is thought to contribute to cartilage degeneration [7]. Synovitis generates inflammatory mediators (TNF-α and IL-1β) that are released into the synovial cavity. We found that moderate exercise of rats injected with MIA not only ameliorated the histological changes but also relieved the damage of collagen II in the cartilage and decreased the levels of these inflammatory mediators in the serum and IALF from these rats. Together, our results corroborate the finding that exercise alleviates damage in the OA knee, which occurs through the suppression of TRAIL, NF-κB p65, and NLRP3 levels in the cartilage.

The mechanisms by which chondrocytes convert biomechanical signals into intracellular events have become an area of intense interest in OA research. Thus, we investigated a cellular model using CTS on chondrocytes. Our previous study showed that CTS (10%, 0.5 Hz) for 4 h alleviated the chondrocyte damage induced by IL-1β [17]. In addition, our findings in the current indicate that CTS administered for different lengths of time could have different effects on TRAIL-induced chondrocytes with 4 h CTS increasing collagen II levels and alleviating chondrocyte damage.

The present findings are consistent with the observations that treadmill exercise causes histological changes in articular cartilage. These findings further suggest that mechanical stimulation is a critical determinant for chondrocytes. Accumulating evidence suggests that TRAIL is a pivotal mediator of OA. The TRAIL signaling pathway can induce apoptosis, leading to joint inflammation and cartilage destruction. Furthermore, we found that the levels of TRAIL increased in OA cartilage. Interestingly, the TRAIL signaling pathway can induce apoptosis in normal cells [26]. Accumulating evidence has demonstrated that articular chondrocytes can be eliminated by apoptosis in OA. The NLRP3 inflammasome produces proinflammatory cytokines, which cause cartilage degeneration and synovial inflammation, and has been implicated in apoptosis in OA [27]. Nuclear translocation of NF-κB p65 is a key event in the activation of this inflammasome [28, 29]. Thus, we investigated the NF-κB/NLRP3 signal on chondrocytes induced by TRAIL.

Intriguingly, the effects of TRAIL on chondrocytes occur through the NF-κB/NLRP3 signaling pathway. TRAIL can inhibit the expression of IκB-α and accelerate NF-κB p65 nuclear translocation. There is also evidence suggesting that the NLRP3 inflammasome is involved in TRAIL-induced chondrocytes, leading to cartilage degradation and synovial inflammation through the activation of NF-κB signaling. Our results showed that TRAIL could also increase ROS generation, which has been shown to activate NLRP3. The NLRP3 inflammasome is composed of NLRP3, adaptor apoptosis-associated speck-like protein containing a CARD, and procaspase-1. NLRP3 ultimately activates caspase-1, which causes maturation of proinflammatory cytokines (e.g., IL-1β) into their active forms and their secretion to further the inflammatory response [30]. IL-1β also stimulates chondrocytes to release MMP-13, which degrades collagen II in the extracellular matrix, leading to a loss of cartilage. Interestingly, NLRP3 is an indicator of pyroptosis, which mainly occurs in the early stage (or acute inflammatory response) of OA [31, 32]. NLRP3 protein levels were significantly higher in the early stage of OA, but NLRP3 would decrease at the end of OA [33].

Importantly, in this study, we found that the NF-κB/NLRP3 signal transduction pathway is central to the effect of CTS. CTS reduced the level of ROS and inhibited NF-κB p65 nuclear translocation via the activation of IκB-α. The decrease in ROS could also inhibit the activation of the NLRP3 inflammasome. Indeed, moderate CTS could inhibit the activation of the NF-κB/NLRP3 signaling pathway and block chondrocyte destruction caused by TRAIL, presumably by abrogating IL-1β production and collagen II breakdown in chondrocytes. Hence, the anti-inflammatory actions of CTS are mediated by the inhibition of both NF-κB nuclear translocation and NLRP3 activation in TRAIL-induced chondrocytes. Moreover, the results of this study

also showed that moderate biomechanical stimulation not only decreased the TRAIL levels in the cartilage but also reduced the sensitization of articular cartilage and chondrocytes to the inflammatory response. These results are consistent with previous studies [24].

This study had some limitations. First, we did not investigate the effects of a variety of CTS conditions on OA. Further study is needed to explore the effects of different CTS conditions (e.g., intensity and frequency) on articular cartilage and chondrocytes. Second, CTS is two-dimensional loading, and chondrocytes are strained in a monolayer in which only one surface is elongated. We will investigate different dimensions of CTS in future studies.

5. Conclusion

In summary, we used treadmill exercise of MIA-induced OA rats and CTS of chondrocytes to explore the effects of mechanical stimulation in OA. Our findings indicated that TRAIL increased during the progression of OA and could damage chondrocytes via the NF-κB/NLRP3 pathway. Moderate mechanical stimulation may reduce the apoptosis of chondrocytes through the inhibition of this pathway. Our results provide not only crucial leads to unveiling the effects of mechanical stress on articular cartilage and chondrocytes but also molecular evidence for biochemical signals generated by mechanical stimulation.

Data Availability

The data used to support the findings of this study have been deposited in the PMC6587477 (doi:10.1002/jcp.27592) and the supplementary information files.

Conflicts of Interest

The authors have declared that no competing interests exist.

Authors' Contributions

Yue Yang, Lunhao Bai, and Feng Li contributed to the conception and design. Yue Yang, Yang Wang, Xiaoning Zhang, Xinyuan Feng, Peng Gao, Ziyuan Wang, and Mingyue Yan are involved in treadmill exercise experiment. Yue Yang, Yang Wang, and He Zhang are involved in cell cultures and cyclic tensile strain. All authors contributed to the acquisition and analysis of data. Yue Yang, Yang Wang, and Yawei Kong are involved in statistical analysis and manuscript preparation. Lunhao Bai conceived the final approval of the version to be submitted and obtaining of funding. All authors contributed in revising the manuscript critically for important intellectual content and approved the manuscript for publication.

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Supplementary Materials

Figure S1: volcano plots and Venn diagram of the DE-miRNAs. (A-C) The black dots represent miRNAs that are not differentially expressed between the cartilage from unexercised or exercised rats for 2 (A), 5 (B), or 15 (C) days. The red and green dots represent the upregulated and downregulated miRNAs, respectively. (D) The Venn diagram of these DE-miRNAs. Figure S2: GO functional annotation analysis. Four categories of GO functional annotation analysis were performed on the potential target genes, including molecular function (A), biological process (B), cellular component (C), and biological pathway (D). Figure S3: protein-protein interaction (PPI) network and KEGG pathway enrichment analysis of target genes. *(Supplementary materials)*

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Research Article

Selenium-Enriched Yeast Alleviates Oxidative Stress-Induced Intestinal Mucosa Disruption in Weaned Pigs

Lei Liu,[1,2] Caimei Wu,[1,2] Daiwen Chen,[1,2] Bing Yu,[1,2] Zhiqing Huang,[1,2] Yuheng Luo,[1,2] Ping Zheng,[1,2] Xiangbing Mao,[1,2] Jie Yu,[1,2] Junqiu Luo,[1,2] Hui Yan,[1,2] and Jun He[1,2]

[1]*Institute of Animal Nutrition, Sichuan Agricultural University, Sichuan Province, Chengdu 611130, China*
[2]*Key Laboratory of Animal Disease-Resistant Nutrition, Sichuan Province, Chengdu 611130, China*

Correspondence should be addressed to Jun He; hejun8067@163.com

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To explore the effect of selenium-enriched yeast (SeY) on intestinal barrier functions in weaned pigs upon oxidative stress, a 2×2 factorial design was utilized and thirty-two pigs were randomly assigned into four groups. Pigs with or without exposure to oxidative stress (diquat challenge) were fed with a basal diet or a SeY-containing diet. The trial lasted for 21 days, and result showed that SeY supplementation attenuated body-weight reduction and significantly decreased the serum concentrations of diamine oxidase (DAO) and D-lactic acid in pigs upon diquat challenge ($P < 0.05$). Diquat challenge decreased the villus height and the ratio of villus height to crypt depth (V/C) in the jejunum and ileum ($P < 0.05$). However, SeY supplementation not only elevated the villus height and the ratio of V/C ($P < 0.05$) but also improved the distribution and abundance of tight-junction protein ZO-1 in the jejunum epithelium. Interestingly, SeY supplementation acutely decreased the total apoptosis rate of intestinal epithelial cells in pigs upon diquat challenge ($P < 0.05$). Moreover, SeY elevated the content of antioxidant molecules such as glutathione peroxidase (GSH-Px) and catalase (CAT) but significantly decreased the content of malondialdehyde (MDA) in the intestinal mucosa ($P < 0.05$). Importantly, SeY elevated the expression levels of critical functional genes such as the nuclear factor erythroid-2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), sodium/glucose cotransporter 1 (SGLT1), and B-cell lymphoma-2 (BCL-2) in the intestinal mucosa upon diquat challenge ($P < 0.05$). Moreover, the expression of caspase-3 was downregulated by SeY in the duodenum and jejunum mucosa ($P < 0.05$). These results indicated that SeY attenuated oxidative stress-induced intestinal mucosa disruption, which was associated with elevated mucosal antioxidative capacity and improved intestinal barrier functions.

1. Introduction

Intestinal barrier mainly consists of a single layer of enterocytes and intercellular tight junctions of enterocytes. It serves as a selective permeable membrane that is not only responsible for nutrient digestion and absorption but also constitutes the first line of defence to prevent a variety of harmful substances from entering the intestinal mucosa and systemic circulation [1]. Moreover, the gastrointestinal tract also serves as the biggest immune organ for animals [2]. A variety of stimuli such as bacterial infections and stresses may impair the integrity of the intestinal barrier. For instance, overproduction of reactive oxygen species (ROS) not only promoted secretion of inflammatory cytokines but also induced intestinal mucosal damage and dysfunction [3]. Evidence is accumulating to show that overproduction of ROS may result in DNA damage and intestinal cell apoptosis, which increases the intestinal permeability and facilitates translocation of luminal antigens into subepithelial tissues leading to a series of intestinal and systemic inflammatory response [4]. Therefore, the avenue to alleviate the ROS-induced disruption of the intestinal barrier has attracted considerable research interest worldwide.

Selenium (Se) is an essential trace element for animals. Importantly, Se is a critical component of the enzyme glutathione peroxidase (GSH-Px), which detoxifies lipid peroxides and provides protection of cellular and subcellular membranes against ROS damage [5]. As compared to

Table 1: Composition and nutrient level of experimental diet.

Ingredients	%	Nutrient level	Contents
Corn	28.31	Digestible energy (calculated, MJ/kg)	14.78
Extruded corn	24.87	Crude protein (%)	19.68
Soybean meal	8.50	Calcium (%)	0.81
Extruded full-fat soybean	10.30	Available phosphorus (%)	0.55
Fish meal	4.20	Lysine	1.35
Whey powder	7.00	Methionine	0.42
Soybean protein concentrate	8.00	Methionine+cysteine	0.60
Soybean oil	2.00	Threonine	0.79
Sucrose	4.00	Tryptophan	0.22
Limestone	0.90		
Dicalcium phosphate	0.50		
NaCl	0.30		
L-Lysine HCl (78%)	0.47		
DL-methionine	0.15		
L-Threonine (98.5%)	0.13		
Tryptophan (98%)	0.03		
Chloride choline	0.10		
Vitamin premix[1]	0.04		
Mineral premix[2]	0.20		
Total	100		

[1]The vitamin premix provided the following per kg of diet: 9000 IU of VA, 3000 IU of VD 3, 20 IU of VE, 3 mg of VK 3, 1.5 mg of VB1, 4 mg of VB 2, 3 mg of VB6, 0.02 mg of VB12, 30 mg of niacin, 15 mg of pantothenic acid, 0.75 mg of folic acid, and 0.1 mg of biotin. [2]The mineral premix provided the following per kg of diet: 100 mg Fe, 6 mg Cu, 100 mg Zn, 4 mg Mn, and 0.30 mg I.

inorganic forms of Se (i.e., sodium selenite and sodium selenate), the Se-enriched yeast (SeY) is mainly consisted of selenoamino acids (i.e., selenomethionine) and their analogues [6]. Previous studies indicated that the SeY was more biologically available than inorganic forms [7]. Importantly, the SeY was found to reduce ROS production and cellular oxidative damage in a variety of animal species [8, 9]. Moreover, the SeY can also act as a clinical health product or drug for various diseases such as Alzheimer's disease [10].

Weaning is a critical developmental stage for neonatal mammals. However, neonatal mammals are more susceptible to various stresses (i.e., oxidative stress) at the weaning stage than other stages because of changes of their living conditions and transition of liquid feed to solid feed. A previous study has indicated that oxidative stress is one of the major causes leading to enterocyte apoptosis and cell cycle arrest in weaning pigs [11]. Pig is an excellent model species used in biomedical researches, as they are closely related to humans in terms of anatomy, genetics, and physiology. Moreover, both species are omnivorous and their organs generally share common functional features [12]. In the present study, we investigated the effect of dietary SeY supplementation on antioxidative capacity and intestinal health in weaned pigs upon oxidative stress. Moreover, the mechanism behind the SeY-modulated intestinal barrier functions has also been explored. This study could also assist in developing of potential clinical health products or drugs for the ROS-induced diseases.

2. Materials and Methods

2.1. Materials. The oxidative stress was induced using diquat injection as described by previous studies [1, 13, 14]. Diquat was purchased from Shanghai Yuanye Biotechnology Co., Ltd., with an average molecular weight of 362.06 and purity of 99%. 10 mg/kg of diquat solution was prepared with sterilized physiological saline and stored at 4-8°C (Shanghai, China) until its use. The SeY was purchased from Sichuan Junzheng Biofeed Co., LTD (organic selenium ≥ 95%).

2.2. Animals and Study Design. Thirty-two weaned pigs (with an average initial body weight 7.30 ± 0.14 kg) were randomly assigned into four treatments consisting of CON (pigs fed with basal diet), SSY (pigs fed with basal diet containing 250 mg/kg SeY), DT (pigs fed with basal diet), and DSY (pigs fed with basal diet containing 250 mg/kg SeY). The trial lasted for 21 d. On 16 d, the CON and SSY groups received intraperitoneal injection of sterile saline (0.9%), whereas the DT and DSY groups received injection of diquat (10 mg/kg BW). All procedures used in the animal experiment were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (no. 20181105). The basal diet (Table 1) was formulated to meet the nutrient requirements recommended by the National Research Council 2012 [15]. Pigs were individually housed in metabolism cages (0.7 m × 1.5 m) and were given ad libitum access to fresh water and feed.

2.3. Sample Collection and Preparation. At the end of the trial, blood samples were collected by venepuncture at 8:00 after 12 h of fasting. Then, the samples were centrifuged at 3500 × g at 4°C for 10 min. After centrifugation, the serum samples were collected and frozen at −20°C until analysis. After blood collection, pigs were euthanized with an intravenous injection of sodium pentobarbital and then slaughtered by exsanguination protocols. Sections of the duodenum, jejunum, and ileum were immediately isolated. Approximately 5 cm segments of the middle of duodenum, jejunum, and ileum were gently flushed with ice-cold phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde solution for morphological analyses and immunofluorescence. Finally, the residual duodenal, jejunal, and ileal segments were scraped with a scalpel blade, and the mucosa samples were collected and stored at −80°C until analysis.

2.4. Serum Biochemical Analysis. The concentrations of D-lactic acid and the activity of diamine oxidase (DAO) in serum were determined using commercially available swine Enzyme-Linked Immunosorbent Assay (ELISA) kits (Jiangsu Jingmei Biotechnology Co., Ltd., Yancheng, China).

2.5. Intestinal Morphology Analysis. About 1 cm segment of the small intestine (duodenum, jejunum, and ileum) was mixed in 10% neutral buffered formaldehyde. The mixed tissue samples were dehydrated with normal saline and then embedded in paraffin. Cross sections of each sample were prepared, stained with haematoxylin and eosin (H&E), and then sealed by a neutral resin size. Ultrathin sections of the duodenal, jejunal, and ileal samples were examined for the villus height and crypt depth with image processing and analysis system (Media Cybernetics, Bethesda, MD, USA). Villus height was calculated from the tip of the villi to the villus-crypt junction. Crypt depth was expressed as the invaginated depth between adjacent villi. A total of 10 intact, well-oriented crypt-villus units were analysed in triplicate per segment. The ratio of villus height to crypt depth (V/C) was calculated from the values described above.

2.6. Immunofluorescence Analysis. The localization of ZO-1 protein in jejunal tissues was determined by immunofluorescence as in the previous method. 4% paraformaldehyde-fixed samples were rinsed in PBS and then incubated with ethylenediaminetetraacetic acid (EDTA, 1 mol/L, pH 9.0, Gooddbio Technology Co., Ltd., Wuhan, China) for antigen retrieval. Tissue sections were blocked with 3% bovine serum albumin prior to incubation with rabbit anti-ZO-1 polyclonal antibody (1 : 250; Abcam Plc., Cambridge, UK) overnight at 4°C. Slides were then washed three times with PBS and incubated with goat anti-rabbit IgG-FITC secondary antibody (Gooddbio Technology Co., Ltd., Wuhan, China) for 1 h at room temperature in the dark. Finally, slides were washed three times with PBS, and the nuclei were stained with $4'$-6-diamidino-2-phenylindole (DAPI, Gooddbio Technology Co., Ltd., Wuhan, China) for 10 min at room temperature in the dark. All slides were examined for fluorescence using a confocal scanning microscope (Nikon Eclipse TI-SR), and images were taken with the NIKON DS-U3 software.

2.7. Flow Cytometry Assays. The percent of jejunum cell apoptosis was determined by flow cytometry. Remove intestinal tissue from the centrifugal tube and flush gently by ice-cold phosphate-buffered saline (PBS: for 1 liter: 8.00 g NaCl, 0.20 g KCl, 1.78 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.27 g KH_2PO_4, pH 7.4), apply the washed intestinal serosa layer to the ice pack, scrape the mucosal cells with a glass slide, cut the tissue block with scissors in the sterile watch glass, add proper RPMI 1640 medium (HyClone, America), then transfer to a new centrifuge tube, and mix the cells on a vortex mixer. Filter the cells in a flow tube with a 300-mesh filter cloth, centrifuge at 300 × g for 5 min, discard the supernatant, and wash again with PBS. Resuspend cells with 200 μL Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and adjust the cell concentration to 10^6 cells/mL with PBS, 4°C. Take 100 μL of cell suspension into a flow tube, resuspend the cells by adding 1 mL of Binding Buffer, centrifuge at 300 × g, 5 min, and discard the supernatant. Add 5 μL Annexin V-FITC (Invitrogen, Australia) staining for fluorescence staining, 10 min, room temperature, and dark; apply 5 μL of PI (propidium iodide) staining solution, 5 min, room temperature; and incubate 500 μL of Binding Buffer. It was detected by CytoFLEX flow cytometry (Beckman, America) with CytExpert software (Beckman, America).

2.8. Enzyme Activity Assays. Several antioxidant-related parameters including catalase (CAT), glutathione (GSH), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) were measured using the assay kits and associated protocols supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.9. RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR. Total RNA was extracted from duodenum, jejunum, and ileum mucosa using the Trizol Reagent (TaKaRa, Dalian, China). Meanwhile, the concentration and purity of total RNA were assayed by a spectrophotometer (NanDrop, Gene Company Limited, Guangzhou, China) at 260 and 280 nm following the manufacturer's guidelines. The ratio of OD 260/280 should vary between 1.8 and 2.0. Reverse transcription using the PrimeScript RT reagent kit (TaKaRa Biotechnology, Dalian, China) was exploited following the manufacturer's instructions. The primers were synthesized commercially by Life Technologies Limited and was exhibited in Table 2.

Quantitative real-time polymerase chain reaction (PCR) was conducted to analyse the mRNA expression abundance of SGLT1, GLUT2, Bcl-2, caspase-3, Nrf2, and HO-1 in the small intestinal mucosa using the CFX-96 real-time PCR detection system (Bio-Rad) and SYBR Premix Ex Taq II (Tli RNaseH Plus) reagents (TaKaRa, Dalian, China). The PCR reaction was run in a 10 μL reaction volume, which contained 5 μL of SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.5 μL of each primer, 1 μL of the cDNA sample, and 3 μL of nuclease-free water. The PCR cycling parameters were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 57.5°C for 30 s, and 72°C for 5 min. A melting curve analysis was performed following each real-time quantitative PCR assay to confirm the gene-specific

Table 2: Primer sequences used for quantitative RT-PCR.

Gene	Accession no.	Primer sequences (5′-3′)	Product length (bp)
Nrf2	XM_003133500.5	GCCCCTGGAAGCGTTAAAC GGACTGTATCCCCAGAAGGTTGT	67
HO-1	NM_001004027	CGCTCCCGAATGAACAC GCTCCTGCACCTCCTC	112
Bcl-2	XM_021099593.1	GCTACTTACTGCCAAAGGGA TTCAGGCGGAGCTGTAAGAG	110
Caspase-3	NM_214131.1	GGAATGGCATGTCGATCTGGT ACTGTCCGTCTCAATCCCA	105
SGLT1	NM_001164021.1	CGCGTCCGGTGTGAAAG CTTCCCGATATCTACACATTCCA	137
GLUT2	NM_001097417.1	GAGGCAGCAGTAGGGAATCTTCGAGCA ACAGTTACTCTGACACCCGTTCTTC	140
β-Actin	XM_003124280.5	TGGAA CGGTG AAGGT GACAGC GCTTTTGGGAAGGCAGGGACT	177

Nrf2: nuclear factor E2-related factor 2; HO-1: heme oxygenase-1; Bcl-2: B-cell lymphoma-2; caspase-3: cysteine aspartic protease-3; SGLT1: sodium glucose transport protein-1; GLUT2: glucose transporter-2.

amplification products had been generated. The housekeeping gene β-actin was used as an internal control for normalization. The target gene mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [16]. Each sample was simultaneously performed on the same PCR plate, and three replicates were set up.

2.10. Statistical Analysis. Data before the injection were analysed by one-way ANOVA. Data after the injection were analysed by two-way ANOVA using the general linear model procedure. Model main effects included SeY and oxidative stress (injection of diquat or saline). Probability values < 0.05 were considered to indicate a significant difference and values between 0.05 and 0.10 to indicate a trend. Variable means for treatments showing significant differences in the ANOVA were separated by Duncan's multiple-range test ($P < 0.05$). Values were expressed as means with their standard errors. All statistical analysis was performed using SPSS 24.0 (SPSS, Inc.).

3. Results

3.1. Effect of SeY on Growth Performance in Weaned Pigs upon Oxidative Stress. As shown in Table 3, there were no significant differences ($P > 0.05$) in average daily food intake (ADFI), average daily gain (ADG), and feed efficiency (G : F) among the four groups from d 1 to d 15 (preinjection). After diquat injection, the ADG and ADFI were significantly reduced in the DT and DSY groups, as compared to those in the CON and SSY groups ($P < 0.05$). However, the ADG and ADFI were higher in the DSY group than those in the DT group ($P < 0.05$).

3.2. Effect of SeY on Serum DAO and D-Lactic Acid Concentrations in Weaned Pigs upon Oxidative Stress. As shown in Figure 1, the serum concentrations of DAO and D-lactic acid were significantly higher in the DT group than those in the CON group ($P < 0.05$). However, the serum concentrations of DAO and D-lactic acid were significantly decreased in the DSY group, as compared to those in the DT group ($P < 0.05$).

3.3. Effect of SeY on Intestinal Morphology and Distribution of the Tight-Junction Protein ZO-1. As compared to the CON group, the villus height in the duodenum and the ratio of V/C in the duodenum, jejunum, and ileum were increased in the SSY group (Table 4 and Figure 2). However, the villus height in the jejunum and ileum was significantly decreased in the DT group ($P < 0.05$). The villus height and the ratio of V/C were higher in the DSY group than those in the DT group ($P < 0.05$). The distribution and abundance of the tight-junction protein ZO-1 are shown in Figure 3. As compared to the CON and DT groups, the localization of ZO-1 in the jejunum was enhanced in the SSY and DSY groups. The ZO-1 staining was diffused with little staining at the intercellular tight junction region in the DT group (indicating disruption of the tight junction).

3.4. Effect of SeY on Apoptosis Rate in Intestinal Epithelial Cell. The late and total apoptosis rates in the jejunal epithelial cells were significantly higher in the DT group than those in the CON group (Figure 4). As compared to the DT group, the late and total apoptosis rates of the jejunal epithelial cells were significantly decreased in the DSY group ($P < 0.05$).

3.5. Effect of SeY on Antioxidant Capacity in the Intestinal Mucosa. As compared to the CON group, the MDA content in the duodenum mucosa was significantly increased in the DT and DSY groups (Table 5). The CAT and T-AOC activities in the duodenum mucosa were higher in the DSY group

Table 3: Effect of SeY on growth performance in weaned pigs upon oxidative stress.

Items	Treatments					*P* value		
	CON	SSY	DT	DSY	SEM	SeY	Diquat	SeY ∗ diquat
Prechallenged (1–15 d)								
ADG (g/day)	340	320	300	320	10			
ADFI (g/day)	410.23	396.90	374.89	38.30	14.62			
F : G	1.20	1.24	1.22	1.21	0.08			
Postchallenged (16–21 d)								
ADG (g/day)	438.33[a]	452.50[a]	-95.83[c]	164.17[b]	63.31	0.03	<0.01	0.048
ADFI (g/day)	511.46[a]	505.25[a]	195.21[c]	311.67[b]	31.69	0.03	<0.01	0.039
F : G	1.17	1.12	—	1.90				

[1]Values are means ± SEM ($n = 6$), nonchallenged pigs (CON, fed with basal diet), diquat-challenged pigs (DT, fed with basal diet), and SeY-treated pigs (fed with basal diet containing 250 mg/kg SeY) challenged by sterile saline (SSY) or diquat (DSY). [2a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$). [3]ADFI = average daily feed intake; ADG = average daily gain; G/F = the ratio of gain to feed intake.

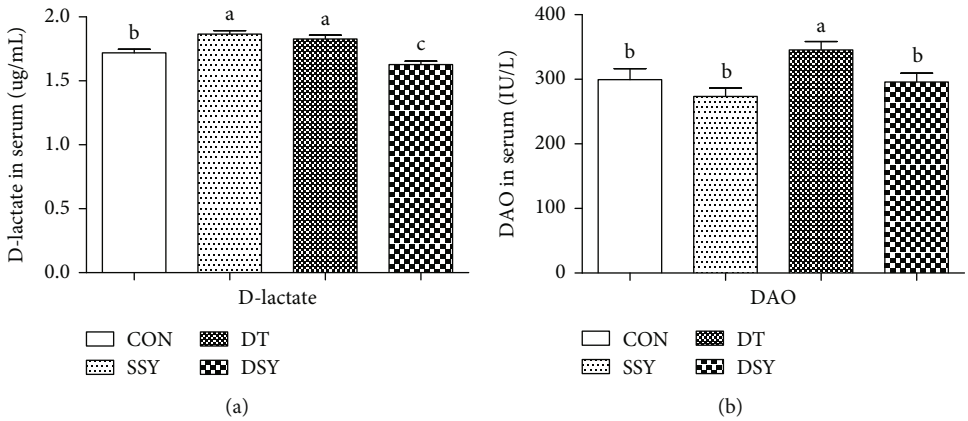

Figure 1: Effect of SeY on serum parameters in weaned pigs upon diquat challenge. [a,b,c]Mean values with different letters on vertical bars indicate significant differences ($P < 0.05$); CON: pigs were fed with basal diet and challenged by sterile saline; SSY: pigs were fed with SeY-containing diet and challenged by sterile saline; DT: pigs were fed with basal diet and challenged by diquat; DSY: pigs were fed with SeY-containing diet and challenged by diquat.

Table 4: Effect of SeY on intestinal morphology in weaned pigs upon oxidative stress.

Items	Treatments					*P* value		
	CON	SSY	DT	DSY	SEM	SeY	Diquat	SeY × diquat
Duodenum								
Villus height (mm)	201.59[b]	228.13[a]	185.31[b]	203.81[b]	26.41	0.08	0.11	0.74
Crypt depth (mm)	87.65	80.39	93.79	83.71	13.23	0.12	0.75	0.87
V/C	2.19 [c]	2.92 [a]	1.99[bc]	2.46[ac]	0.5	0.01	0.11	0.52
Jejunum								
Villus height (mm)	144.27[ab]	181.36[a]	124.18[c]	157.89[a]	5.98	<0.01	0.03	0.84
Crypt depth (mm)	67.73	55.83	67.88	63.81	2.62	0.14	0.44	0.46
V/C	2.15[bc]	3.27[a]	1.87[c]	2.57[b]	0.15	<0.01	0.02	0.28
Ileum								
Villus height (mm)	155.54[a]	196.36[a]	142.29[b]	175.86[a]	8.47	0.18	0.15	0.51
Crypt depth (mm)	66.49[ab]	59.11[b]	75.34[a]	56.38[b]	2.59	<0.01	0.45	0.51
V/C	2.29[bc]	3.21[a]	1.91[b]	2.66[ac]	0.15	<0.01	0.01	0.61

[1]Values are means ± SEM ($n = 6$), nonchallenged pigs (CON, fed with basal diet), diquat-challenged pigs (DT, fed with basal diet), and SeY-treated pigs (fed with basal diet containing 250 mg/kg SeY) challenged by sterile saline (SSY) or diquat (DSY). [2a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

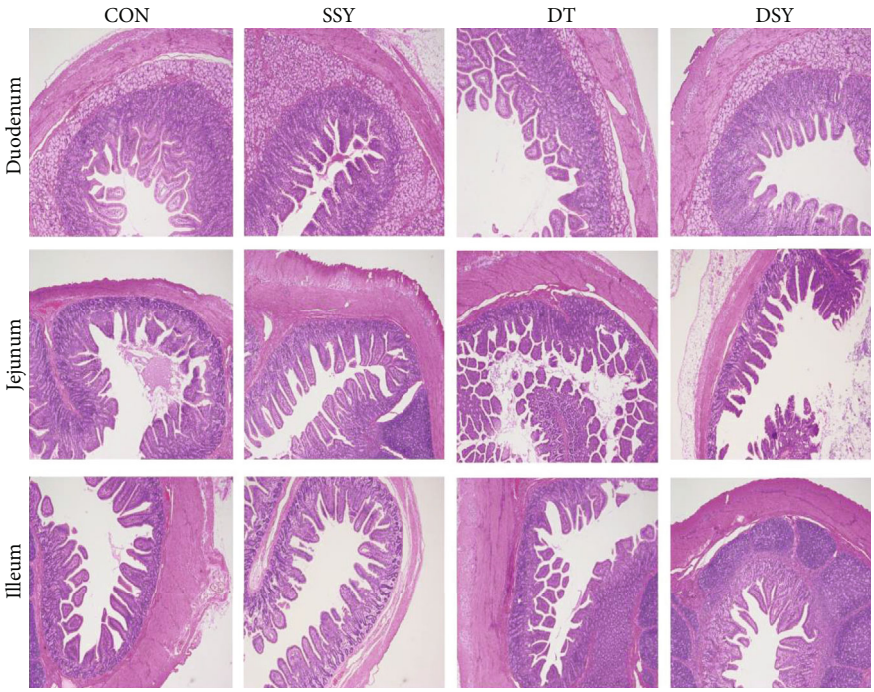

Figure 2: Effect of SeY on small intestinal morphology in weaned pigs (H&E; ×40). CON: pigs were fed with basal diet and challenged by sterile saline; SSY: pigs were fed with SeY-containing diet and challenged by sterile saline; DT: pigs were fed with basal diet and challenged by diquat; DSY: pigs were fed with SeY-containing diet and challenged by diquat.

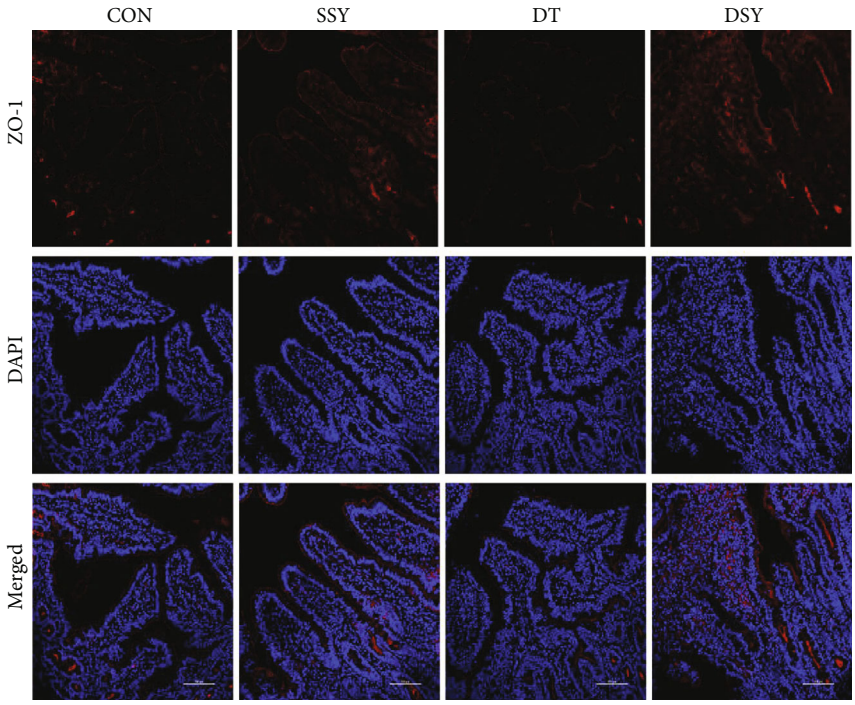

Figure 3: Effect of SeY on tight junction distribution and localization of ZO-1 in the intestinal epithelium. Localization of ZO-1 and DAPI (DNA) within the jejunum-weaned pigs was assessed by immunofluorescence. ZO-1 protein (red), DAPI stain (blue), and merged ZO-1 protein and DAPI are presented. CON: pigs were fed with basal diet and challenged by sterile saline; SSY: pigs were fed with SeY-containing diet and challenged by sterile saline; DT: pigs were fed with basal diet and challenged by diquat; DSY: pigs were fed with SeY-containing diet and challenged by diquat.

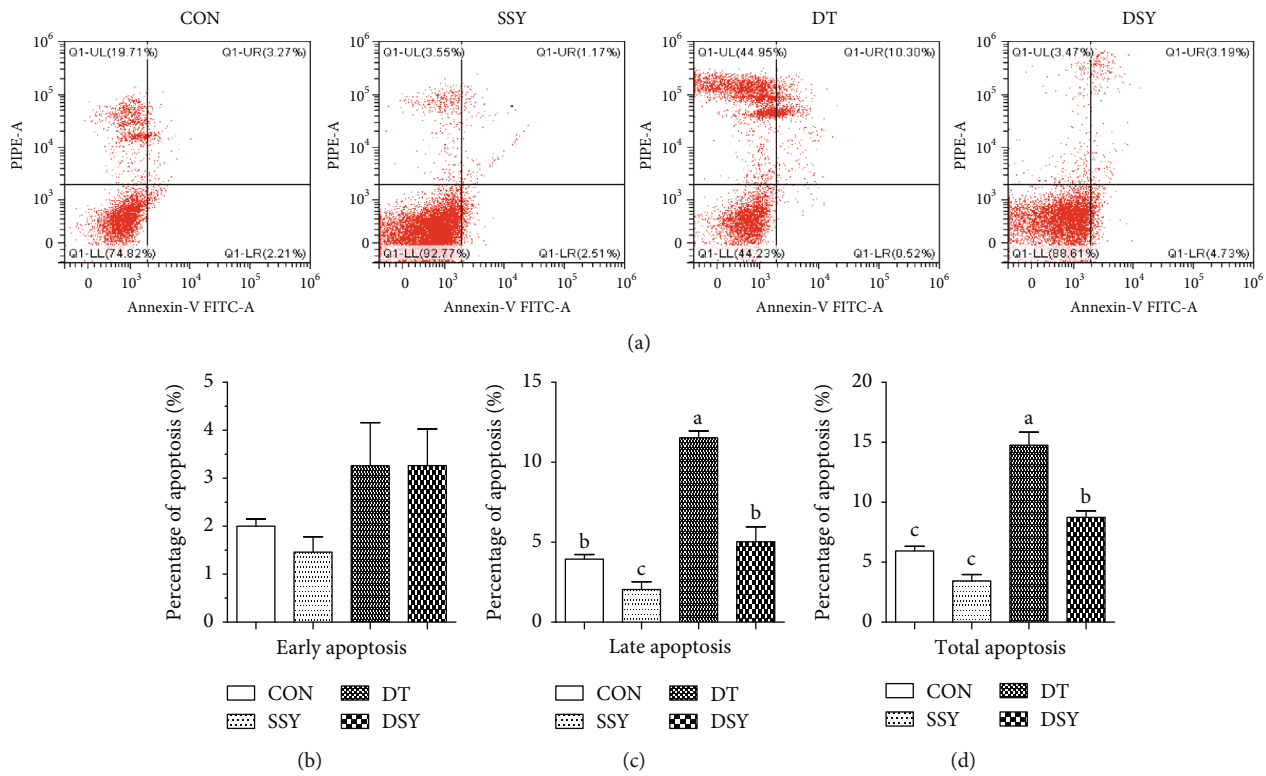

Figure 4: Effect of SeY on apoptosis rate in intestinal epithelial cells. (a) Evaluation of jejunal cell apoptosis by flow cytometry in weaned pigs exposed to SeY and diquat challenged. 30,000 cells were used in each acquisition reading. Frames were divided into 4 quadrants: Q1-UL represents necrotic cells; Q1-UR represents late apoptotic and early necrotic cells; Q1-LR represents early apoptotic cells; and Q1-LL represents normal cells; percentages of apoptotic cells of early apoptosis (b), late apoptosis (c), and total apoptosis (d) in the jejunum, respectively. [a,b,c]Mean values with different letters on vertical bars indicate significant differences ($P < 0.05$). CON: pigs were fed with basal diet and challenged by sterile saline; SSY: pigs were fed with SeY-containing diet and challenged by sterile saline; DT: pigs were fed with basal diet and challenged by diquat; DSY: pigs were fed with SeY-containing diet and challenged by diquat.

Table 5: Effect of SeY on antioxidant capacity of the intestinal mucosa.

Items	Treatments					*P* value		
	CON	SSY	DT	DSY	SEM	SeY	Diquat	SeY × diquat
Duodenum								
GSH-Px (mg/gprot)	56.61	58.61	66.28	78.6	2.66	0.46	0.03	0.73
T-AOC (U/mgprot)	0.49^{ac}	0.78^{ac}	0.45^{bc}	0.92^{a}	0.37	0.02	0.76	0.54
MDA (nmol/mL)	0.23^{c}	0.43^{bc}	0.92^{a}	0.62^{b}	0.07	0.59	<0.01	0.03
CAT (U/gprot)	15.50^{b}	20.02^{b}	20.42^{b}	30.78^{a}	1.84	0.02	0.02	0.32
Jejunum								
GSH-Px (mg/gprot)	95.36^{a}	92.79^{a}	72.41^{b}	97.41^{a}	3.39	0.09	0.09	0.04
T-AOC (U/mgprot)	1.50^{ac}	1.33^{a}	0.80^{bc}	1.30^{a}	0.39	0.23	0.02	0.02
MDA (nmol/mL)	1.87^{a}	1.43^{ac}	1.78^{a}	1.14^{b}	0.47	0.04	0.83	0.40
CAT (U/gprot)	33.5	28.52	30.56	33.34	1.32	0.69	0.73	0.17
Ileum								
GSH-Px (mg/gprot)	76.74^{ab}	85.00^{a}	71.13^{b}	95.49^{a}	3.64	0.02	0.71	0.23
T-AOC (U/mgprot)	0.24^{bc}	0.29^{ac}	0.22^{bc}	0.41^{a}	0.13	0.04	0.35	0.19
MDA (nmol/mL)	0.71^{a}	0.59^{b}	1.38^{a}	0.57^{b}	0.58	0.09	0.34	0.03
CAT (U/gprot)	8.58^{b}	8.24^{b}	6.40^{b}	12.17^{a}	0.59	<0.01	0.25	<0.01

[1]Values are means ± SEM ($n = 6$), nonchallenged pigs (CON, fed with basal diet), diquat-challenged pigs (DT, fed with basal diet), and SeY-treated pigs (fed with basal diet containing 250 mg/kg SeY) challenged by sterile saline (SSY) or diquat (DSY). [2a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$). [3]GSH-Px: glutathione peroxidase; T-AOC: total antioxidant capacity; MDA: malondialdehyde; CAT: catalase.

than in the DT group ($P < 0.05$). Moreover, the GSH-Px and T-AOC activities in the jejunum and ileum mucosa were also higher in the DSY group than those in the DT group ($P < 0.05$).

3.6. Effect of SeY on Expressions of Critical Genes Related to Nutrient Absorption and Intestinal Barrier Functions. The expression levels of SGLT1 in the intestinal mucosa were higher in the DSY group than those in the DT group ($P < 0.05$; Figure 5). As compared to the CON group, SeY supplementation not only elevated the expression level of SGLT1 in the jejunum mucosa but also elevated the expression level of GLUT1 in the ileum mucosa ($P < 0.05$). Moreover, we found that SeY supplementation significantly elevated the expression levels of ZO-1 in the duodenum and jejunum mucosa in pigs upon oxidative stress ($P < 0.05$). As compared to the DT group, the expression levels of Bcl-1, Nrf-2, and HO-1 in the duodenum and jejunum mucosa were higher in the DSY group than those in the DT group ($P < 0.05$). In contrast, the expression levels of caspase-3 in the duodenum and jejunum mucosa were significantly decreased in the DSY group, as compared to those in the DT group ($P < 0.05$).

4. Discussion

Weaning is a critical developmental stage for mammals. At this stage, animals are more susceptible to exogenous stresses including the oxidative stress because of alterations of their living conditions [17, 18]. A previous study indicated that oxidative stress is one of the major contributors leading to growth retardation and intestinal mucosa disruption in neonatal animals including the pigs [11]. Selenium (Se) is an essential trace element for pigs. Importantly, Se is a critical component of the enzyme glutathione peroxidase (GSH-Px), which detoxifies lipid peroxides and provides protection of cellular and subcellular membranes against ROS damage [5]. In the present study, we investigated the effect of dietary SeY supplementation on growth performance and intestinal health in weaned pigs upon oxidative stress. The oxidative model was induced by using diquat injection, which is a well-established method [1, 13, 14]. We found that the ADG and ADFI were decreased in pigs upon diquat injection (DY and DSY). The result is consistent with a previous study on the weaned pigs that oxidative stress led to significant reduction of the growth performance [19]. However, SeY supplementation significantly attenuated the body-weight reduction in DYS after diquat injection. The result is consistent with previous studies on pigs and broilers that dietary SeY supplementation could improve their growth performance and elevate their antioxidative capacity [20, 21].

A previous study suggested that oxidative stress suppresses the intestinal barrier functions by inducing apoptosis of the intestinal epithelial cells [4]. DAO is a highly active intracellular enzyme in all mammalian intestinal mucosal epithelial cells, and the DAO activity in serum can act as a marker of intestinal epithelial cell maturity, integrity, and functional status [22]. D-lactic acid is a metabolite fermented by the gastrointestinal tract bacteria [23], and few of them can be absorbed into the blood under normal condition. However, a large amount of D-lactic acid can be absorbed into the blood circulation system through the impaired intestinal mucosa [24, 25]. In the present study, the serum concentrations of DAO and D-lactic acid were significantly higher in the DT group than those in the CON group, indicating disruption of the intestinal epithelial barrier upon diquat injection. However, both their concentrations were significantly decreased in the DSY group, as compared to those in the DT group.

An integrated intestinal morphological structure is of great importance for digestion and absorption [26]. In the present study, the villus height in the jejunum and ileum was decreased in the DT group, as compared to that in the CON group. This is consistent with a previous study that overproduction of ROS may lead to disruption of the intestinal morphology such as villus atrophy and crypt hyperplasia [27]. However, the villus height was higher in the DSY group than that in the DT group. Previous study indicated that the intestinal epithelial barrier is maintained and regulated by the tight junctions between the epithelial cells [28]. ZO-1 is one of the most important tight-junction proteins, which directly or indirectly anchors to the actin-based cytoskeleton and then forms a selective permeable barrier [29]. In the present study, the ZO-1 protein was highly expressed and localized in the apical intercellular region of the intestinal epithelium in the DSY group. Both these results suggested a protective effect of the SeY on the intestinal barrier in pigs upon oxidative stress.

Cell apoptosis is a physiological process that plays a vital role in maintaining the intestinal epithelial turnover [30]. However, excessive apoptosis disrupts the homeostasis of the intestinal epithelial cells, which may lead to mucosa atrophy and other intestinal disorders [31]. In the present study, the total apoptosis rate of the jejunal epithelial cells was higher in the diquat challenged pigs (DT and DSY) than that in the nonchallenged pigs (CON and SSY). The result is consistent with previous studies that overproduction of ROS can induce DNA damage and apoptosis in a variety of animal species and cell lines [32]. As compared to the DT group, the total apoptosis rate was significantly decreased in the DSY group. This may be attributed to the elevated antioxidative capacity in the intestinal mucosa [33]. A previous study indicated that diquat can directly catalyze the molecular oxygen to produce O^{-2} and H_2O_2, which initiates the lipid peroxidation and produces a large number of free radicals [34, 35]. Moreover, the SeY was reported to have the potentials to serve as a therapeutic antioxidant agent by reducing lipid peroxidation and ROS production, weakening DNA oxidative damage, and inhibiting cell apoptosis [8, 9]. In the present study, SeY supplementation significantly elevated the content of GSH-Px and CAT in the intestinal mucosa, which offers sufficient antioxidant molecules to abolish the free radicals.

We also explored the expression levels of several critical genes involved in the intestinal barrier functions. The SGLT1 is an active glucose transporter that takes up glucose into cells independent of the extracellular concentration of glucose. It

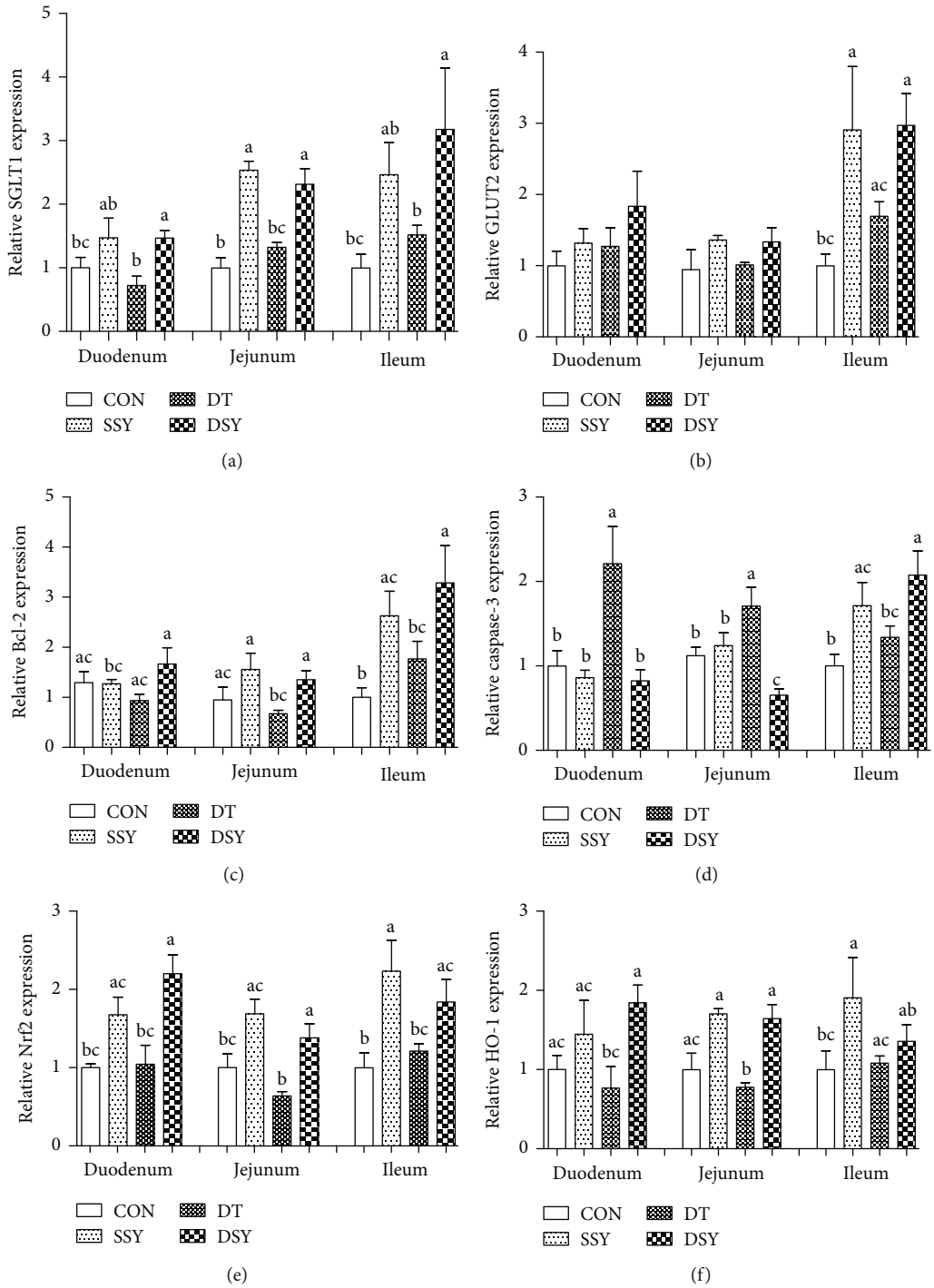

Figure 5: Relative expression levels of critical genes involved in the intestinal barrier functions. SGLT1: sodium glucose transport protein-1 (a); GLUT2: glucose transporter-2 (b); Bcl-2: B-cell lymphoma-2 (c); caspase-3: cysteine aspartic protease-3 (d); Nrf2: nuclear factor E2-related factor 2 (e); HO-1: heme oxygenase-1 (f). [a,b,c]Mean values with different letters on vertical bars indicate significant differences ($P < 0.05$). CON: pigs were fed with basal diet and challenged by sterile saline; SSY: pigs were fed with SeY-containing diet and challenged by sterile saline; DT: pigs were fed with basal diet and challenged by diquat; DSY: pigs were fed with SeY-containing diet and challenged by diquat.

plays a critical role in maintaining glucose homeostasis at both physiological and pathological levels [36]. In the present study, the expression level of the SGLT1 in the intestinal mucosa was higher in the DSY group than that in the DT group, indicating an improved absorption ability by SeY supplementation. The Bcl-2 and caspase-3 are two critical molecules involved in the regulation of apoptosis [37]. The Bcl-2 is a negative regulator of apoptosis, which can protect many

types of cells from apoptosis [38], while the caspase-3 can act as one of the most important initiator caspases that are closely coupled to proapoptotic signals [39]. Once activated, initiation caspases cleave and activate downstream effector caspases (i.e., caspase-3), which in turn execute apoptosis by cleaving targeted cellular proteins [40]. In the diquat challenged pigs (DT and DSY), SeY supplementation significantly improved the expression level of Bcl-2 but downregulated the expression of caspase-3, which offers molecular basis for the decreased cell apoptosis in the jejunal epithelium. Moreover, the expression levels of two critical genes involved in the antioxidative signaling were determined. The Nrf2 is a critical transcription factor that can regulate genes involved in the production of a wide variety of antioxidant enzymes (i.e., glutathione and catalase) and detoxification or "stress-response" genes [41]. The HO-1 is one of the most important target genes of Nrf2, which can catalyze the rate-limiting step in heme degradation and produce free iron, biliverdin, and carbon monoxide. Importantly, the HO-1 has been implicated in the regulation of a series of biological processes including inflammation, apoptosis, fibrosis, and angiogenesis [42]. In the present study, their expression levels were higher in the DSY group than those in the DT group. The result is in accordance with the measurements of the antioxidative enzymes in the intestinal mucosa. Both results indicated that the SeY can act as a potential therapeutic antioxidant agent.

5. Conclusions

The present study suggested that dietary SeY supplementation can attenuate the growth retardation of the weaned pigs in response to the oxidative stress. Moreover, SeY alleviates the oxidative stress-induced disruption of the intestinal mucosa, which was associated with elevated mucosal antioxidative capacity and improved intestinal barrier functions. The beneficial effects of SeY supplementation on the growth and intestinal health upon oxidative stress suggested that it can serve as a potential therapeutic antioxidant agent.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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