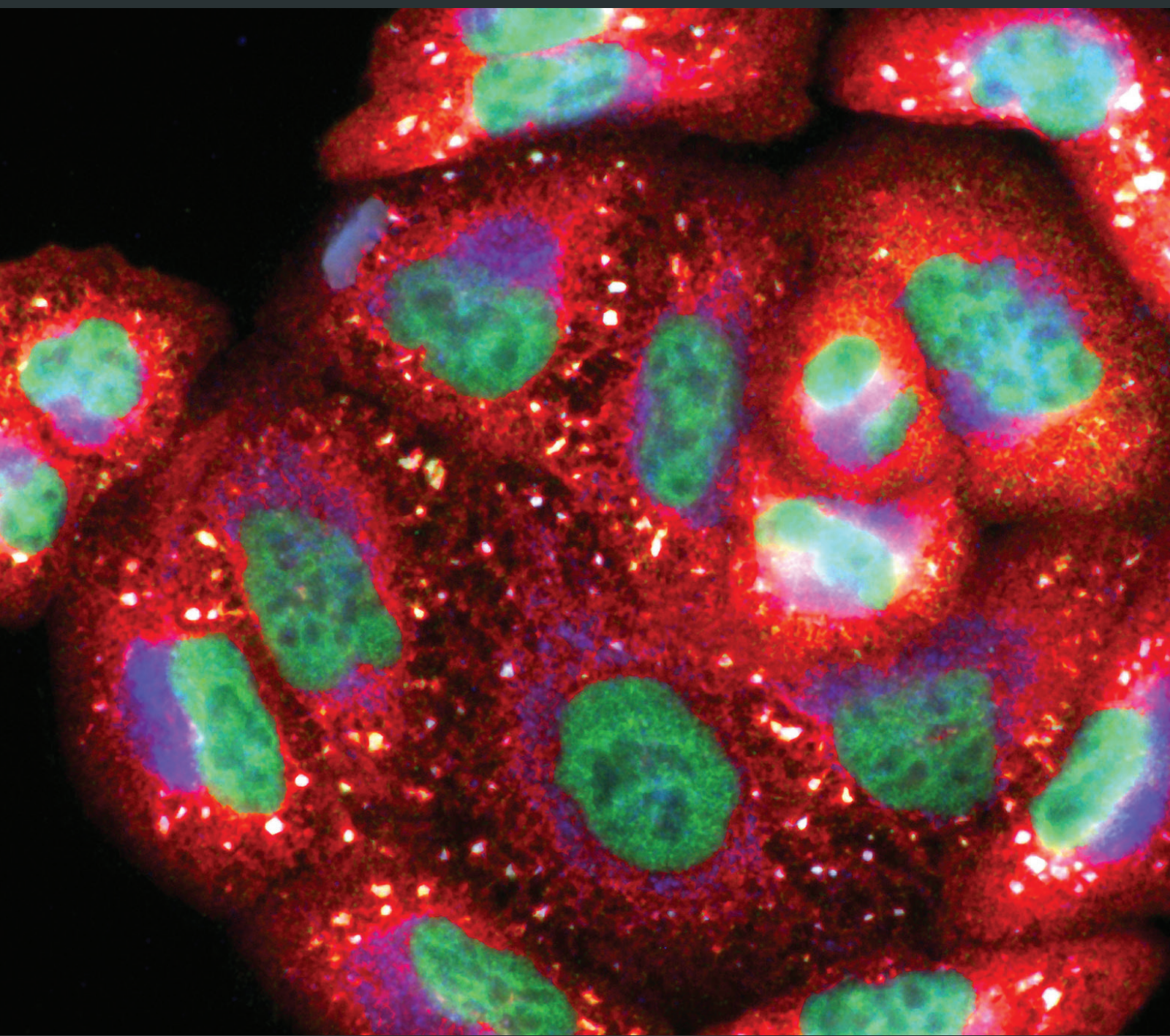
Oxidative Medicine and Cellular Longevity

Reactive Oxygen Species in Cancer Biology and Anticancer Therapy

Guest Editors: Alexandr V. Bazhin, Pavel P. Philippov, and Svetlana Karakhanova



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Editorial Board

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4197815, 2 pages
http://dx.doi.org/10.1155/2016/4197815

Editorial

Reactive Oxygen Species in Cancer Biology and Anticancer Therapy

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Received 12 September 2016; Accepted 19 September 2016

Our understanding of reactive oxygen species (ROS)—a group of highly reactive chemicals containing oxygen—has changed in the last few years from ROS as just harmful substances to crucial intra- and extracellular messengers as well as important regulators controlling a wide spectrum of signaling pathways. Nevertheless, there are still many uninvestigated points and open questions regarding ROS, especially in pathophysiology. Delicately controlled ROS homeostasis is critical for maintaining normal cell functions and any disruption in the oxidation-antioxidation balance leads to oxidative stress associated with a wide spectrum of human disorders such as chronic inflammation, age-related diseases, and cancers. In health, the intracellular ROS level is tightly controlled by various antioxidants. In contrast, cancer cells have an abnormally high level of ROS due to an increased ROS production and/or impaired ROS detoxification that can damage intracellular macromolecules such as nucleic acids, proteins, and lipids. Elevated ROS production in cancer cells may result from an aberrant metabolic activity, mitochondrial dysfunction, disturbed cellular signaling, oncogene activity, and interaction with tumor infiltrating immune cells.

The ultimate purpose of this special issue is to publish high-quality research communications as well as review articles dedicated to the role of ROS in cancer biology, anticancer therapy, and related topics. Five articles published in this special issue are devoted to reactive oxygen species in cancer biology. Presently, it is rather well known that H_2O_2 has the opposite effects on cancer cell proliferation depending on its concentration and cancer type. G. Vilema-Enriquez et al. in their article "Molecular and Cellular Effects of Hydrogen Peroxide on Human Lung Cancer Cells: Potential Therapeutic Implications" review effects of hydrogen peroxide on human lung cancer. The authors discussed effects of H_2O_2 on migration and invasion, calcium release, and other molecular features of cancer cells. Furthermore, they describe the link between hydrogen peroxide and inflammation. Finally, the authors hypothesize that novel therapeutic approaches against lung cancer may be based on the use of H_2O_2. Y.-C. Hung et al. in their review "Roles of Reactive Oxygen Species in Anticancer Therapy with *Salvia miltiorrhiza* Bunge" deal with Danshen as a drug of the traditional Chinese medicine and provide a systematic review of its antioxidant capacity and potential anticancer effects. Moreover, they conclude that based on the existed preclinical data this drug may be pipelined in clinical trials. A research paper by W. Li et al. ("Hyperglycemia Promotes the Epithelial-Mesenchymal Transition of Pancreatic Cancer via Hydrogen Peroxide") deals with hyperglycemia in pancreatic cancer cells. The authors succeeded in finding the link between hyperglycemia and epithelial-mesenchymal transition through the production of hydrogen peroxide. Another research report on breast cancer of D. M. Badr et al. ("The Combination of α-Tocopheryl Succinate and Sodium Selenite on Breast Cancer: A Merit or a Demerit?") shows in vitro and in vivo that

sodium selenite antagonizes effects of α-tocopheryl succinate on apoptosis induction in cancer cells via inhibition of oxidative stress. An intriguing review came from France, authored by M. Assi and A. Rébillard, and was devoted to the problem of cachexia in cancer patients ("The Janus-Faced Role of Antioxidants in Cancer Cachexia: New Insights on the Established Concepts"). As regulators of catabolic pathways ROS are involved in muscle atrophy in cachectic cancer patients, the authors summarize and discuss contradictory data on the effects of antioxidants in such patients.

The next topic highlighted in this issue is devoted to ROS in tumor immunology. A review by X. Chen et al. ("Reactive Oxygen Species Regulate T Cell Immune Response in the Tumor Microenvironment") gives readers an overview of ROS in the tumor microenvironment and especially in the tumor-induced immunosuppression. The authors, based on improvement of anticancer T cell response, consider an antioxidant treatment as a promising option for cancer therapy. A. Scala et al. in their research article "Alterations in Red Blood Cell Functionality Induced by an Indole Scaffold Containing a Y-Iminodiketo Moiety: Potential Antiproliferative Conditions" deal with a prediction of the antiproliferative effects of heterocyclic scaffolds, which could be important for development of new therapeutic approaches against cancer.

A research article by M. Weniger et al. ("The Analgesic Effect of the Mitochondria-Targeted Antioxidant SkQ1 in Pancreatic Inflammation") considers pancreatitis as a main risk factor for pancreatic cancer. The authors show an unexpected analgesic effect of the new antioxidant SkQ1 during pancreatic inflammation. The last article from this issue deals with the oxidative stress in cancer-prone diseases in pediatric age. S. Perrone et al. in "Oxidative Stress in Cancer-Prone Genetic Diseases in Pediatric Age: The Role of Mitochondrial Dysfunction" review recent literature on such diseases and discuss molecular mechanisms of oxidative stress associated with mitochondrial dysfunction. They conclude that mitochondria-targeted medicines could be applied into the clinics to improve the quality of life of patients with cancer-prone genetic diseases.

Summarizing, the wide spectrum of review and research articles presented in this issue provides recent interesting data on ROS in the context of cancer biology and anticancer therapy.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2104247, 11 pages
http://dx.doi.org/10.1155/2016/2104247

Research Article

Alterations in Red Blood Cell Functionality Induced by an Indole Scaffold Containing a Y-Iminodiketo Moiety: Potential Antiproliferative Conditions

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Received 17 February 2016; Revised 6 July 2016; Accepted 11 July 2016

Academic Editor: Svetlana Karakhanova

We have recently proposed a new erythrocyte-based model of study to predict the antiproliferative effects of selected heterocyclic scaffolds. Starting from the metabolic similarity between erythrocytes and cancer cells, we have demonstrated how the metabolic derangement induced by an indolone-based compound (DPIT) could be related to its antiproliferative effects. In order to prove the validity of our biochemical approach, in the present study the effects on erythrocyte functionality of its chemical precursor (PID), whose synthesis we reported, were investigated. The influence of the tested compound on band 3 protein (B3), oxidative state, ATP efflux, caspase 3, metabolism, intracellular pH, and Ca^{2+} homeostasis has been evaluated. PID crosses the membrane localizing into the cytosol, increases anion exchange, induces direct caspase activation, shifts the erythrocytes towards an oxidative state, and releases less ATP than in normal conditions. Analysis of phosphatidylserine externalization shows that PID slightly induces apoptosis. Our findings indicate that, due to its unique features, erythrocyte responses to exogenous molecular stimuli can be fruitfully correlated at structurally more complex cells, such as cancer cells. Overall, our work indicates that erythrocyte is a powerful study tool to elucidate the biochemical/biological effects of selected heterocycles opening considerable perspectives in the field of drug discovery.

1. Introduction

Red blood cells (RBCs) are by far the most abundant cells in the blood and the simplest cells found in mammals. Due to the uniqueness of the direct relationship with each type of cell soma and owing to a metabolism greatly limited compared to other cells, RBC has become an unmatched and efficient model of scientific studies in biochemical and clinical researches [1, 2]. Its availability, the easy handling and preparation, and its natural "dispersion" in buffered aqueous solvents make it suitable to study the effects of exogenous substances on its functionality. The RBCs responses to exogenous molecular stimuli, if properly evaluated, can clarify their intriguing and seemingly simple metabolism and, at the same time, they can also be profitably correlated at structurally more complex cells, such as neoplastic cells.

In this scenario, we have recently proposed an unprecedented erythrocyte-based biochemical approach focused on the metabolic similarity between cancer cells and RBCs to predict the antiproliferative effects of heterocyclic scaffolds [3]. Thus, we have investigated how the metabolic derangement of RBCs induced by DPIT (2,2′-dimethyl-6,6′-diphenyl-6,6′,7,7′-tetrahydro-H,1′H-2,3′-biindole-3,4,4′(2H, 5H,5′H)-trione) (Figure 1), selected as an indole-based *model compound*, could be related to its antiproliferative effects [4, 5]. With the aim to demonstrate the versatility and applicability of our approach, we herein further expand our studies focusing on PID (Figure 1), the chemical precursor of

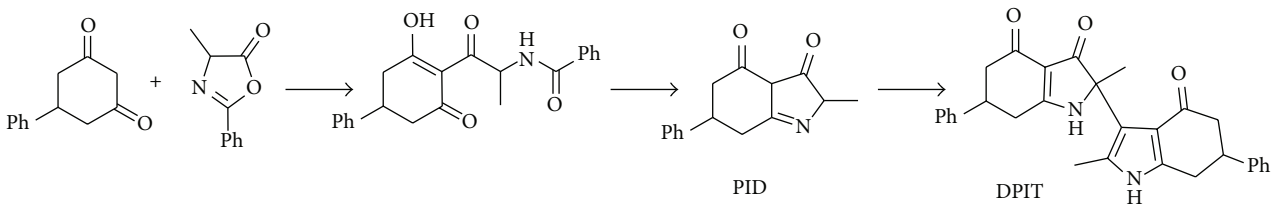

Figure 1: Multistep synthesis of PID.

DPIT. PID is an unprecedented indole-3,4-dione synthesized by some of us *via* one-pot acid-promoted *N*-deprotection-cyclization of the corresponding 1,3,3′-tricarbonyl precursor, powerful intermediate bearing an intriguing triketo Y-topology, the latter being obtained by microwave-mediated nucleophilic addition of 5-phenyl-1,3-cyclohexanedione to 4-methyl-2-phenyl-oxazol-5-one (Figure 1) [4, 6, 7]. Actually, our interest in the chemistry of both enolizable cyclic 1,3-diketones and azlactones as building blocks for the synthesis of novel molecular architectures is well documented [4–15]. PID is a small weight heterocycle functionalized with the nitrogen analogue of the Y-triketo moiety, which could experience prototropic changes and bestows on it fascinating properties, such as an intrinsic stability and ability to act as bidentate chelating ligand.

Within our ongoing effort to propose new *N,O*-heterocycles with useful biological properties [7, 12–14], congeners of PID have been recently evaluated *in vitro* for antiviral activity against herpes simplex virus type-1 (HSV-1), resulting in lack of cytotoxicity and significant antiproliferative activity [7]. Nowadays we became interested in exploring the effect of PID on RBC functionalities, because we supposed that it could be able to cross the erythrocyte membrane, unlike its precursor, due to its smaller molecular weight, and consequently it could induce a pronounced metabolic derangement, one of whose most striking manifestations is the caspase 3 activation.

Caspase 3 belongs to a family of cysteine aspartate proteases responsible for degradation of cellular proteins and for the triggering of the apoptosis cell suicide program. It is a dormient proenzyme maintained in an inactive structural conformation, by an Asp-Asp-Asp regulatory tripeptide named "safety catch" [16]. This tripeptide is kept by *in situ* ionic interactions highly sensitive to pH that are disrupted by intracellular acidification, resulting in enhanced autocatalytic maturation of the protein that becomes more available to proteolytic activation. Resistance of caspase 3 activation plays a critical role in determining the sensitivity of cells to apoptosis and thus may contribute to the attenuated apoptosis observed in many cancers. Indeed, neoplastic cells have been shown to sequester caspase 3 in its inactive form, and thus, therapies that focus on activating caspase 3 are a promising novel anticancer strategy. The "safety catch" therefore is an important regulatory checkpoint that precludes the accidental activation of procaspase 3 in healthy cells having stable pH_i, while facilitating proteolytic activation of caspase 3 in damaged or stressed cells in which homeostatic maintenance of normal pH_i is perturbed [16].

The primary cellular targets of caspase 3 are the cytoplasmic domain of the B3 (cdB3), the Na^+/H^+ exchanger (NHE1), and the 4 plasma membrane Ca^{2+}-ATPase (PMCA4) [17–19]. As it is known, cdB3 has several functions as the maintenance of anion homeostasis, the cytoskeleton cell shape, and the regulation of the metabolic glucose 6 phosphate pathways. In detail, cdB3 competitively binds both hemoglobin (Hb) and a number of glycolytic enzymes (GE). The cleavage of cdB3 induced by caspase 3 activation causes a preferential channeling of glucose 6 phosphate (G6P) in the Embden-Meyerhof pathway (EMP) at the expense of the pentose phosphate pathway (PPP). Consequently both the increased lactate production by EMP and the lack of NADPH lead to cytosolic acidification and increase of oxidative stress [20].

NHE1 is a member of a family of electroneutral exchangers ubiquitously expressed that play an essential role in the regulation of pH_i, protection against cytosolic acidification, and absorption of HCO_3^- [21]. NHE1, activated by a decrease in pH_i, mediates the exchange of intracellular H^+ with extracellular Na^+, while HCO_3^- comes out from B3 in exchange for Cl^-. NHE could be affected by numerous endogenous and exogenous stimuli and in diverse pathological situations; it has also been shown to play an important role in the proliferation disorders [22, 23].

Taking into account that intracellular alkalinization is a common feature of proliferative processes [24], Izumi et al. rightfully proposed that the induction of intracellular acidification using, for example, pharmacological inhibitors of the NHE might serve as a therapeutic tool for treating some types of cancer [25].

Pászty et al. identified an additional cleavage target of caspase 3 on the PMCA, a calmodulin-regulated Ca^{2+} pump driven by ATP expressed in the plasma membrane of all eukaryotic cells [19]. Among the multiple isoforms of PMCA, 1 and 4 are typical of RBCs [26]. PMCA4 cleavage causes irreversible activation of the Ca^{2+} transport activity of the enzyme [27]. Several studies have suggested that changes in intracellular Ca^{2+} homeostasis play an important role in apoptosis [28, 29]. Indeed, the primary switch in the decision between necrosis and apoptosis depends on various factors, including the intensity of the insult, the degree of the initial Ca^{2+} overload, and the intracellular ATP levels [30]. Deregulated apoptosis has been implicated in the development of many pathologic conditions, including neurodegenerative disorders, autoimmune diseases, sepsis, and particularly cancer [31, 32]. In this context, it is now widely acknowledged that evasion of apoptosis is one of the hallmarks of cancer development, and naturally, this discovery has led to a diverse

array of scientific explorations to identify drug targets and develop compounds that might effectively treat cancer through restoration of the apoptotic program [33–36].

Altogether these considerations prompted us to investigate the influence of PID on RBC functionalities, with particular reference to caspase activation, B3, oxidative state, intracellular ATP concentration and transport, metabolism, intracellular pH, and Ca^{2+} homeostasis with the aim to point out that the metabolic derangements induced in RBC by PID would be unfavorable to the life cycle of neoplastic cells.

2. Materials and Methods

2.1. Reagents and Compounds. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Citrate fresh human blood was obtained from informed healthy donors who declared that they had abstained from all drug treatment for at least one week prior to sample collection, in accordance with the principles outlined in the Declaration of Helsinki. Concentrated stock solution was prepared by dissolving PID in dimethyl sulfoxide (DMSO). PID was synthesized as previously described [4].

2.2. Preparation of Erythrocytes. Citrate blood samples were washed three times with an isoosmotic NaCl solution and treated as previously reported [37].

2.3. High Performance Liquid Chromatography (HPLC) Determinations. Washed RBCs were incubated at 37°C for 2 h with PID (100 μM) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM HEPES [N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid], and 1.5 mM $MgCl_2$), adjusted to pH 7.4. Samples were washed and the packed cells were lysed with 10% ethanol. Lysates were centrifuged at 4000 ×g for 10 min at 4°C and the supernatant was filtered with 0.45 μm filter. Free PID was analyzed by HPLC with a Shimadzu system, consisting of an LC-10AD pomp system and an SPDM10A diode array detector, a Rheodyne 7725i injector with a 20 μL sample loop, and a reverse-phase Supelco C18 column (5 mm, 250 × 4.6 mm). The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 5–20% (0–2 min), 20–30% (2–4 min), 30–100% (4–7 min), and 100% (7–10 min). The flow rate was 1.0 mL/min at 25°C. PID was detected at 286 nm and determined by comparison of peak areas with a standard solution of PID (100 μM). To establish the amount of PID in the membrane bilayer, we induced RBCs hemolysis with hypotonic shock and centrifuged the samples at 15000 rpm for 15 min at 4°C. The supernatant was removed and the packed membrane structures were washed and centrifuged, as described above, three times with isoosmotic NaCl solution to eliminate the unbounded compound. After that, the packed membranes were treated with DMSO for 2 h and analyzed by HPLC to identify and quantify PID.

2.4. Binding to Hb. Purified Hb (0.7 mg/mL) in the T or R state was incubated for 1 h at 37°C in 0.1 M HEPES buffer plus 0.1 M NaCl with PID (100 μM) and 0.3 M 2,3-biphosphoglyceric acid at pH 7.4. The free PID has been separated from the one bound to hemoglobin utilizing Microcon YM 30 (Nominal Molecular Weight Limit 30,000), filtered with 0.45 μm filter, and analyzed by HPLC as described above.

2.5. Met-Hemoglobin (Met-Hb) Determination. Washed RBCs were treated with PID (100 μM), at different incubation times from 6 to 24 h, lysed with distilled water and freezing at −20°C, and then centrifuged at 18000 rpm for 30 min. The percentage of met-Hb was determined spectrophotometrically in a range of wavelength from 500 to 680 nm.

2.6. Metal Chelating Activity. The chelation of Fe^{2+} by PID (0–100 μM) was estimated by method of Dinis et al. [38]. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_0] \times 100$, where A_0 was the absorbance of the control and A_s was the absorbance of the samples in the presence of PID (562 nm).

2.7. Band 3 Anion Exchanger Activity Determination: Sulphate Transport Measurement. Cells were incubated in the incubation buffer containing sulphate at 25°C, in the presence and absence of PID (100 μM). At specified intervals 10 μmol of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) stopping medium was added to each test tube containing the RBC suspension. Cells were separated from the incubation medium by centrifugation (J2-HS Centrifuge, Beckman, Palo Alto, CA, USA) and washed three times at 4°C with a sulphate-free medium. After the final washing, the packed cells were lysed with perchloric acid (4%) and distilled water and centrifuged at 4°C. Sulphate ions in the supernatant were precipitated by adding glycerol and distilled water (1 : 1), 4 M NaCl and 1 M HCl solution, and 1.23 M $BaCl_2 \cdot 2H_2O$ to obtain a homogeneous barium sulphate precipitate. The intracellular sulphate concentration was measured by spectrophotometry at 425 nm wavelength as reported previously [39].

2.8. Determination of Phosphatase PTP-1B Activity. Cells were incubated in the incubation buffer at 37°C in the presence and absence of PID (100 μM) and treated as previously reported [40].

2.9. Effects on Superoxide Anion Generation. Superoxide anions were measured as previously reported [41].

2.10. Reduced Glutathione (GSH) Measurements. GSH was analyzed in haemolysate using the Ellman method [42]. The samples were treated with trichloroacetic acid (TCA) and the protein precipitate was removed by centrifugation. The concentration of GSH was estimated in mmol/packed cells (PC).

2.11. Total Thiols Measurements. The content of the total thiols was measured using the method of Ellman [42]. Samples were diluted with a 20 mmol/L phosphate buffer,

pH 8.0, containing SDS. Following this, DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) from a 10 mmol/L stock solution was added and samples were incubated for 1 h at 37°C. The thiols reacted with DTNB to form anions with a strong yellow color which were optically active at 412 nm. The basal optical activity of the samples was measured before the addition of DTNB. A calibration curve was prepared using different concentrations of GSH. The concentration of the thiol groups was calculated and expressed as μmol/mg proteins of plasma or as nmol/mg proteins of RBC membranes.

2.12. Glutathione Peroxidase (GPx) Analysis. GPx activity inside the RBC was analyzed by a commercial kit (Glutathione Peroxidase Cellular Activity Assay Kit, Sigma-Aldrich) following the instruction supplied by the seller.

2.13. Lipid Peroxidation Assay. Isolated RBCs were incubated for 2 h in the absence or in the presence of PID (25, 50, and 100 μM) and analyzed as previously described [3].

2.14. Acetylcholinesterase (AChE) Enzyme Assay. AChE activity was assayed in RBCs suspensions after PID (100 μM) treatment using the colorimetric method proposed by Ellman et al. [43].

2.15. Measurement of Percentage Haemolysis. The haemolysis of RBCs was determined spectrophotometrically at 576 nm based on the ratio of Hb released from cells to the total cellular Hb content after haemolysis with distilled water. The ratio of haemolysis was calculated from the following equation: $H(\%) = A_1/A_2 \times 100\%$, where $H(\%)$ is the percent of haemolysis of the RBCs, A_1 is the absorbance of the supernatants of the samples of the RBCs incubated with or without PID (100 μM), and A_2 is the absorbance of the supernatant of the samples after complete haemolysis with distilled water.

2.16. Caspase 3 Assay. Citrate blood samples were washed three times with an isoosmotic NaCl solution and treated as previously reported [41], using PID (50 μM) and tert-butylhydroperoxide (t-BHT), 100 μM.

2.17. pH_i Measurement. Isolated RBCs were incubated from 2 to 24 h in the absence or in the presence of PID (100 μM). After incubation, the samples were washed 3 times with 10 volumes of isoosmotic NaCl and lysed by treatment with ice distilled water, vortex, and ultrasonication. Then the samples were centrifuged and the pH_i was measured using a pH meter ProLab 3000 Schott.

2.18. Annexin V Apoptosis Detection. Fluorescence-activated cell sorting (FACS) analysis was performed as described by Andree et al. [44]. RBCs were incubated for 6, 12, and 24 h in the presence or absence of PID (100 μM) in annexin-binding buffer containing 0.14 M NaCl, 0.01 M HEPES-NaOH (pH 7.4), and 2.5 mM $CaCl_2$. RBCs were suspended in a solution composed of Annexin-V-Fluos and annexin buffer. After 10 min of incubation in the dark, samples were finally diluted 1 : 5 in annexin-binding buffer and measured using flow cytometric analysis. Cells were analyzed by forward scatter, and annexin fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

2.19. Measurement of Intra-/Extracellular Ca^{2+}. Isolated RBCs were incubated for 2 and 6 h in the absence or in the presence of PID (100 μM). After incubation, the samples were washed 3 times with 10 volumes of isoosmotic NaCl and centrifuged at 2500 rpm for 5 min. Then, intra-/extracellular Ca^{2+} concentration was analyzed by a commercial kit (Calcium Colorimeric Assay Kit, Sigma-Aldrich) following the instruction supplied by the seller.

2.20. Measurement of Intra-/Extracellular ATP. ATP was measured by the luciferin-luciferase technique, as previously reported [45].

2.21. Statistical Analysis. Data are presented as mean of four different experiments ± standard deviation (SD). The data were analyzed by one-way analysis of variance. The significance of the differences in relation to the respective controls for each experimental test condition was calculated by Student's t-test for each paired experiment. A P value of <0.05 versus control was regarded as significant difference and indicated with asterisks in the figures.

3. Results and Discussion

3.1. PID Crosses the RBC Membrane. HPLC observations of RBCs incubated with PID (100 μM) at 37°C for 2 h reveal that PID crosses the RBC membrane reaching inside of the cell a 20% share (Figure 2). The different molecular weight could probably explain the greater ability of PID to cross the plasma membrane, localizing in the cytosol, with respect to the precursor DPIT. According to our previous reports [36, 40, 41, 46, 47], we can extrapolate that exogenous compounds permeate more easily through the RBC membrane within the molecular weight range 200–300 g/mol.

3.2. PID Does Not Bind Hb but Increases the B3 Protein Exchange. Since Hb and B3 are the two most abundant RBC proteins, inside the cytoplasm and in the membrane, respectively, the effects of PID on their structure and functionality were explored.

To this end, purified Hb was incubated with PID (100 μM) for 1 h at 37°C and the levels of free PID were assessed by HPLC, excluding PID-Hb interaction (data not shown). Furthermore it does not affect the Hb redox reactions because no increased values of met-Hb were registered incubating RBCs with PID (100 μM) for 6-12-24 h (data not shown). Additionally, the inability of PID to chelate Fe^{2+} was demonstrated by UV-vis spectroscopy.

The influence of PID on B3 was studied evaluating spectrophotometrically its effect on anion exchanger functionality after pretreatment of RBCs with PID (100 μM) and

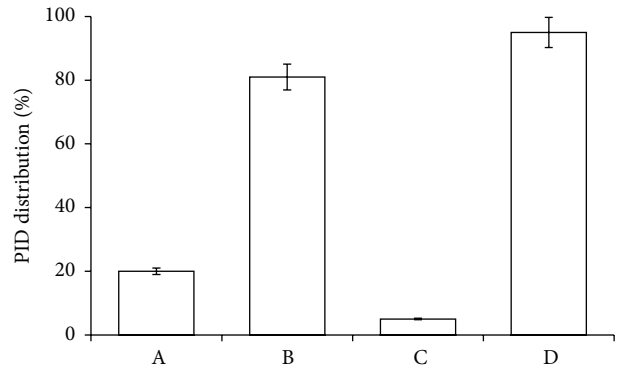

Figure 2: HPLC determination: PID distribution inside (A), outside (B), and in the RBC membrane (C), compared to the control (PID 100 μM standard solution (D)).

comparing the results with the control. Figure 3(a) shows an increase of anion exchange of about 30% in the presence of PID (rate constant: 0.017 and 0.012 min^{-1} in RBCs incubated with and without PID, resp.).

The derangement of B3 function, being one of the main causes of the pH_i decrease, could act as a factor which creates an "acidic environment" for organ cells. Since pH_i has been shown to be alkaline in many human cancer cells and to be an important trigger for cell proliferation [24], PID influence on B3 functionality could contribute to inhibiting cell proliferation and leading the tumor cells to be more sensitive to antitumor drug. Also the fact that B3 interacts with and regulates the function of p16, a key negative regulating protein for the cell cycle [48], is not to be underestimated. In this context Shen et al. demonstrated that B3 plays a crucial role in the pathogenesis of gastric and colonic adenocarcinoma and that p16 dysfunction is a novel pathway of carcinogenesis [49]. To find potential justification for PID-induced destabilization on B3 physiological exchange, we tested the tyrosine phosphatase activity as an index of phosphorylation state of RBC (Figure 3(b)). It is noteworthy that changes in phosphorylation are among the most important modulations of protein activity in RBCs [50]. In particular, the delicate balance between phosphorylation and dephosphorylation on RBC membrane depends on the action of two types of proteins, tyrosine phosphatases (PTP1B) and src tyrosine kinases that are strongly influenced by free radical concentration [51].

Thus, the PTP1B activity was tested in the presence of PID (100 μM) or orthovanadate (OV), a known phosphatase inhibitor. Results shown in Figure 3(b) highlighted that PID induced hyperactivation of phosphatases (about 30%) in comparison to the control, clearly indicating an alteration of RBC phosphorylation balance.

The phosphorylation and consequent inhibition of the pyruvate dehydrogenase complex (PDC) would contribute to the Warburg metabolic correlated with malignant progression of cancer cells [52]. Taking into account the metabolic similarities between RBCs and cancer cells that we have recently proposed [3], we can speculate that the correction of this metabolic abnormality could offer opportunities for cancer treatment and may potentially synergize with other cancer therapies.

3.3. PID Influences the RBC Oxidative State. The influence of PID on the redox equilibrium of the RBCs was evaluated in terms of superoxide generation, GSH levels, GPx activity, thiol redox status, and lipid peroxidation. The rate of superoxide generation was analyzed *in vitro* at different concentrations (10, 25, 50, and 100 μM), resulting in the fact that PID triggered superoxide generation at 50 μM and more evidently at 100 μM (Figure 4).

GSH is a principal intracellular thiol-containing compound and it is involved in maintaining the oxidation-reducing balance in RBCs. Therefore GSH concentration and thiol redox status have been evaluated in RBCs pretreated with PID (100 μM). Figure 5 shows the depletion of GSH (a) and the decrease of -SH groups (b), in comparison to the control. Furthermore, the GPx activity was also tested, showing that PID does not alter the enzyme functionality (data not shown).

Oxidation of -SH groups is strictly related with lipid oxidation of the membrane. Then peroxidation was evaluated on the RBC membrane after incubation for 2 h with PID (25-50-100 μM). Unexpectedly a slight inhibition at the higher concentration was observed, compared with the control (Figure 6), likely due to the ability of PID to break the lipid peroxidation chain reaction.

Such considerations are further supported by the evaluation of the integrity of plasma membrane assessed monitoring the functionality of AChE, a well-known marker of cell membrane wholeness, resulting in the fact that PID (0–100 μM) did not significantly modify the enzyme activity (data not shown). Moreover, it did not increase the percentage of haemolysis.

Based on the experimental evidences, we assume that PID shifts the RBCs towards an oxidative state, increasing the generation of superoxide and the oxidation of thiol groups. Additionally, reduced GSH levels are detected, leading to dangerous oxidant/antioxidant imbalance and to an increase of intracellular H_2O_2. Indeed GSH, through the action of GPx, catalytically detoxifies the cells from peroxides such as H_2O_2. So the GSH depletion always causes accumulation of reactive oxygen species (ROS) and consequently intracellular acidification [24, 53].

3.4. PID Influences Caspase 3 Activation and pH_i. Generally, the increase of the oxidative stress and the decrease of pH significantly contribute to the direct activation of caspase 3 by removal of the "safety catch" [16, 54–56]. To confirm the above, RBCs were incubated, respectively, in the absence and in the presence of PID (50 μM) or t-BHT (100 μM) as a reference oxidant. Figure 7 shows that PID significantly induces caspase 3 activation, even superior to t-BHT.

Generally, caspase 3 activation leads to inappropriate triggering or rapid disablement of key structural proteins and important signaling, homeostatic and repair enzymes [57]. In nucleate cells, caspase 3 processing occurs in a protease cascade involving mitochondrial release of cytochrome c in the

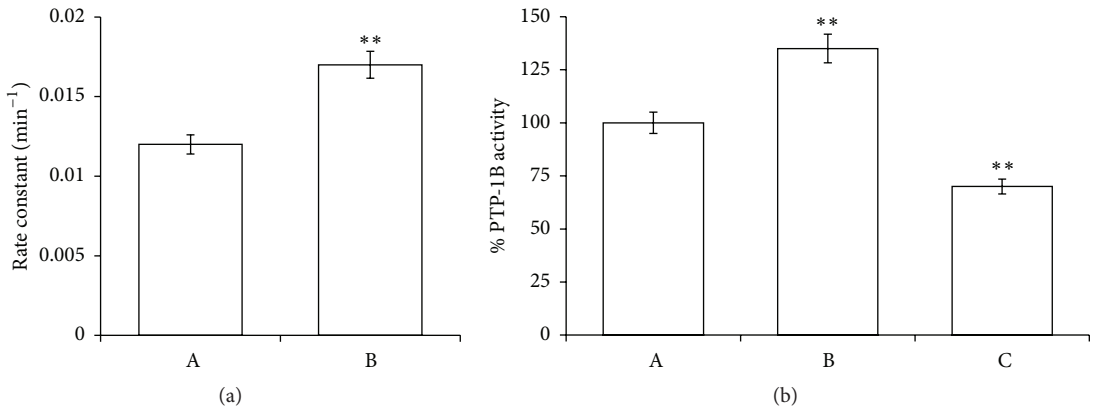

Figure 3: Effects of PID on rates of sulphate transport (a) and on phosphatase activity (b) in normal human RBCs, incubated in absence (A) or in the presence of 100 PID μM (B) or OV 1.0 mM (C). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ versus control.

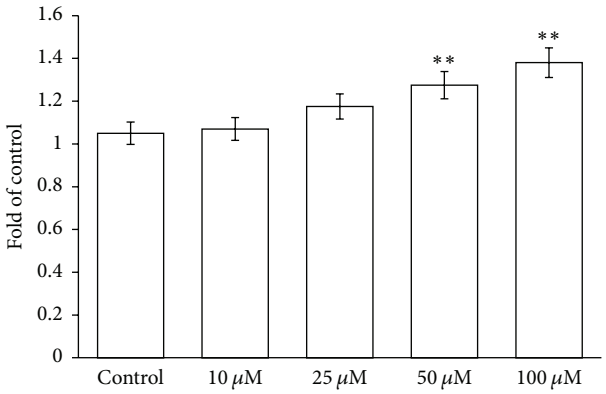

Figure 4: Effects of PID (0–100 μM) on superoxide anion radical generation. Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ versus control.

cytosol, while in RBCs, in the absence of mitochondria and cytochrome c, this mechanism appears to operate directly. Caspase 3 catalyzes the specific cleavage of cdB3, NHE1, and PMCA4. The cdB3 and NHE1 cleavage contributes to the alteration of the hydrogen ions concentration, as HCO_3^-/Cl^- exchange occurs in conjugation with the Na^+/H^+ antiporter [18]. The hyperstimulation of the B3 induced by PID should be offset by the NHE1 activity to maintain the correct pH homeostasis. However, caspase 3 activation results in NHE1 inhibition and reduced Na^+/H^+ antiporter activity acidifies cells [18, 58]. Therefore, PID would change the pH_i of RBCs inducing cytosolic acidification, according to literature [59]. To confirm the above, pH_i was measured by incubating RBCs in the presence of PID (100 μM), resulting in a decrease of 0.1 units. Literature data have recently reported that intracellular acidification in mammalian cells, typically amounting to 0.3–0.4 pH_i units, can be detected following exposure of cells to external stimuli as UV irradiation, staurosporine, and etoposide [24]. Our experimental observation, namely, a variation of 0.1 pH_i units could be related to the presence in RBCs of a high concentration of Hb that can buffer a more pronounced cytosolic acidification. Indeed, Hb, at a concentration of 7 mmoles per litre of cell water, is the RBC's main proton buffer [60].

Furthermore, within the last decade, numerous studies have demonstrated that pH_i homeostasis is often dramatically altered in cancer cells, as they maintain a pH_i more alkaline than their normal counterparts [61]. This has sparked substantial interest in pH regulation as a potential therapeutic target relevant to many forms of cancers [62]. In particular, regulation of pH_i may be a possible mechanism for tumor-selective therapy. Rightfully, it has even been proposed that the induction of an intracellular acidification, using, for example, pharmacological inhibitors of the NHE, might serve as a therapeutic tool for treating some types of cancer. In this context, we postulate that PID may determine in cancer cells a more pronounced cytosolic acidification with respect to that observed in our "buffered" erythrocyte-based model of study, providing a way of inducing tumor-specific apoptosis, thus aiding cancer chemotherapy.

3.5. PID Influences Intracellular Ca^{2+} Homeostasis, ATP Efflux, and RBC Metabolism. Generally, a decrease in pH_i is the initial trigger for a cascade of events resulting in apoptosis [25]. Indeed, acidification facilitates the caspase 3 activation by removal of the "safety catch", that in turn has been shown to induce phosphatidylserine (PS) exposure [63]. In normal RBCs, plasma membranes exhibit significant phospholipid asymmetry, with phosphatidylcholine and sphingomyelin predominantly on the external side and phosphatidylethanolamine and PS on the inner side. Entry into apoptosis leads to a loss of phospholipid asymmetry, with exposure of PS on the outer side. It was shown that the anticoagulant annexin V preferentially binds to negatively charged phospholipids like PS. Thus, this binding of annexin V was used to detect PS exposure on the membrane of apoptotic cells in cytofluorimetric assays (Figure 8). Our experiments were performed at 6, 12, and 24 h, resulting in a 4.2% of apoptosis in the early phase (6 h), while at longer

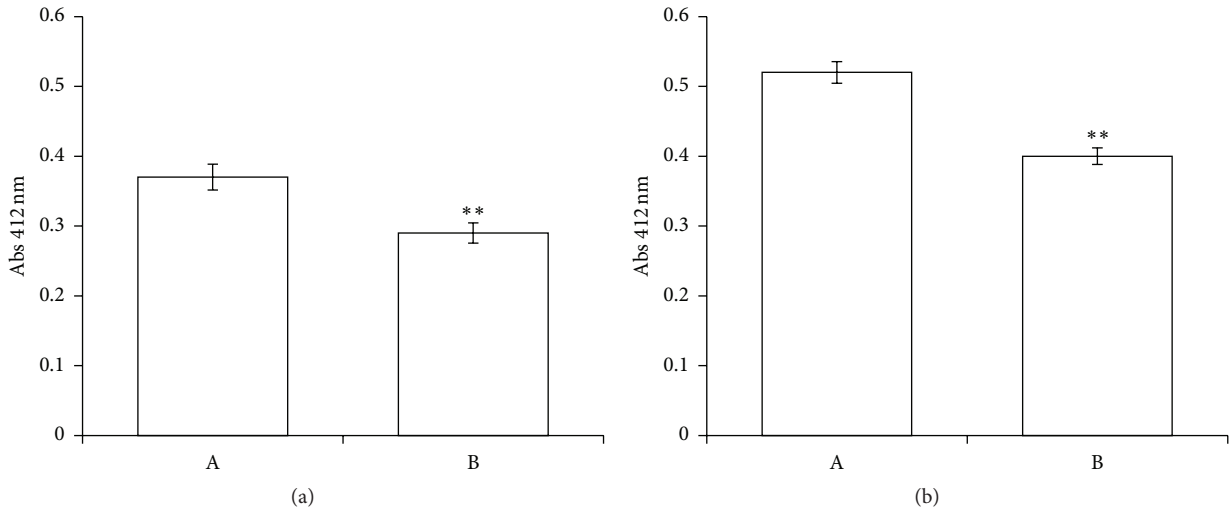

Figure 5: Influence of PID on intracellular levels of GSH (a) and total thiols (b). The RBCs were incubated for 2 h in absence (A) or in the presence of PID 100 μM (B). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ versus control.

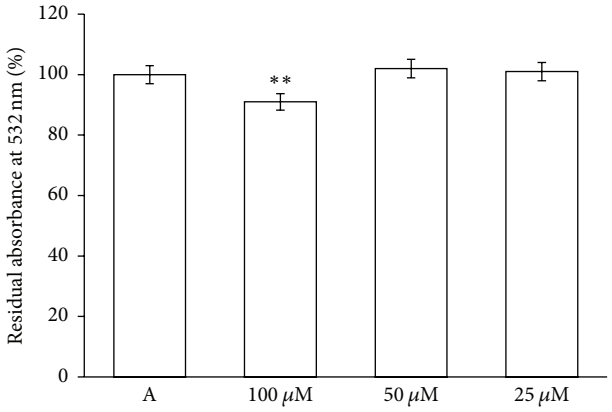

Figure 6: Influence of PID on lipid peroxidation of RBC membrane. The RBCs were incubated for 2 h in absence (A) or in the presence of PID (25–100 μM). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ versus control.

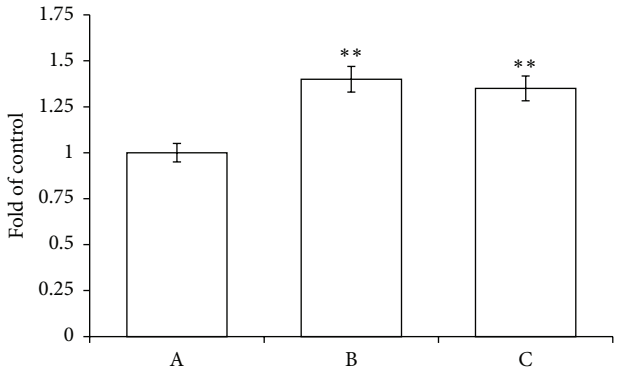

Figure 7: Caspase 3 activity in RBCs in the absence (A) and in the presence of PID 50 μM (B) or t-BHT 100 μM (C). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ versus control.

incubation periods the intensity of the apoptotic process increases (4.8% at 12 h; 8.2% at 24 h).

Triggers of apoptosis include exposure to several stressors such as oxidative stress, NHE inhibitors, cytosolic acidification, and increase of cytosolic Ca^{2+} levels [64]. In this regard, the effects of PID (100 μM) on the calcium-permeable channels PMCA were evaluated at 2 h and 6 h, showing a slight increase in the intracellular free Ca^{2+} levels (data not shown), in accordance with the low apoptotic effect observed.

The limiting factor of the PMCA transport capacity is ATP availability [50]. Indeed, both the Ca^{2+} homeostasis and the cellular ATP are important determinants of cell death. In particular, cells remain alive when certain level of ATP is maintained, but when ATP falls below this level, apoptosis is activated, and a severe drop in cellular ATP causes cell necrosis [65]. Thus, the influence of PID on ATP release from RBCs was evaluated, showing that RBCs pretreated with PID (100 μM) released significantly less ATP than in normal conditions, but the intracellular [ATP] does not appear affected by the treatment (Figure 9), although both the PMCA4 hyperactivity and the phosphatases triggering should deplete the cellular ATP.

We suggest that this condition could be related to an alteration of the metabolic modulation of RBCs attributable to PID influence. In particular, as cdB3 serves as a docking station for multiple GE, its cleavage operated by caspase 3 deprives RBCs of the fundamental and primary regulation of metabolic G6P pathways. Specifically, the predominant EMP is favored to produce ATP and NADH, at the expense of the PPP, only source of reducing power (NADPH). Cancer

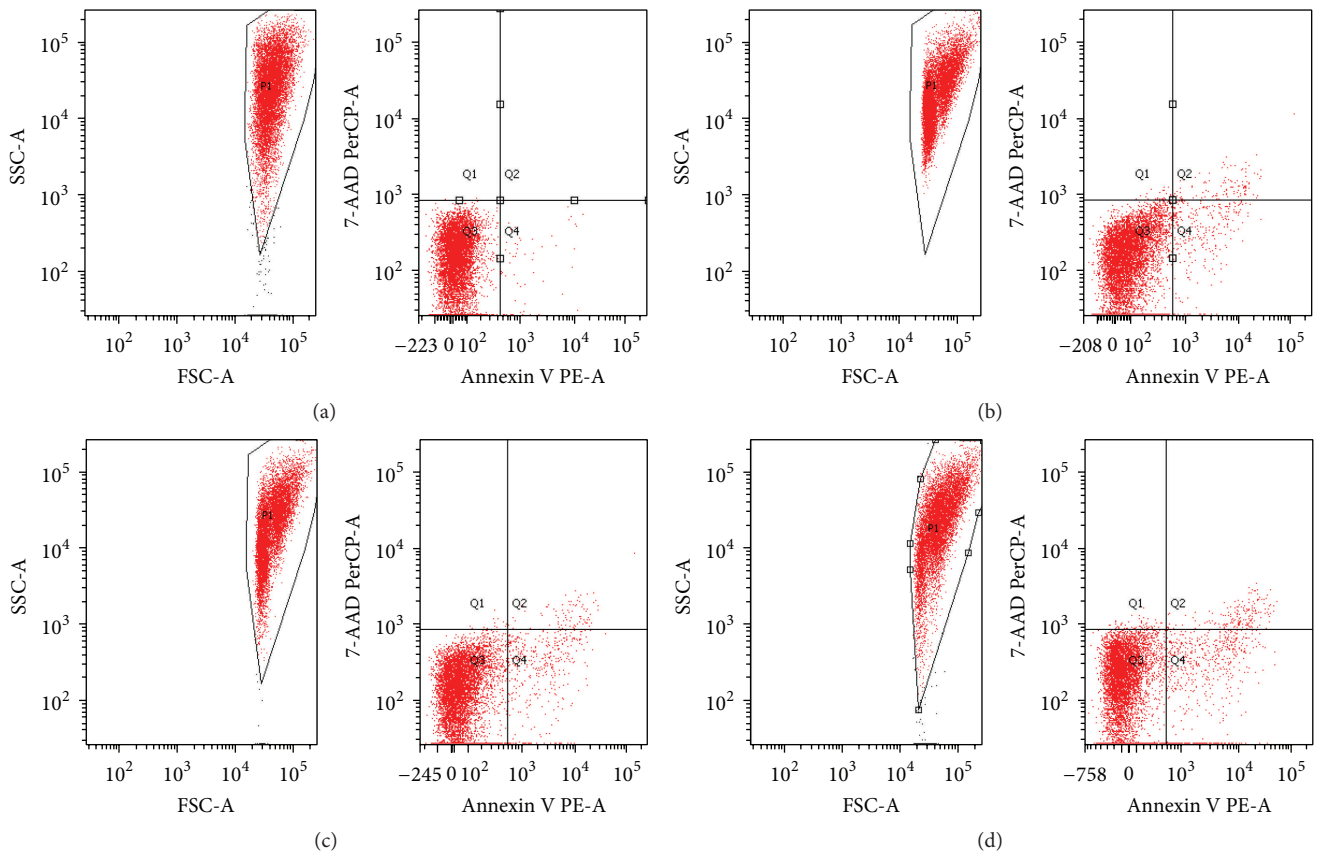

FIGURE 8: Flow cytometric analysis of apoptosis in RBC in the absence (a) and in the presence of PID (100 μM) after 6 h (b), 12 h (c), and 24 h (d) of incubation time.

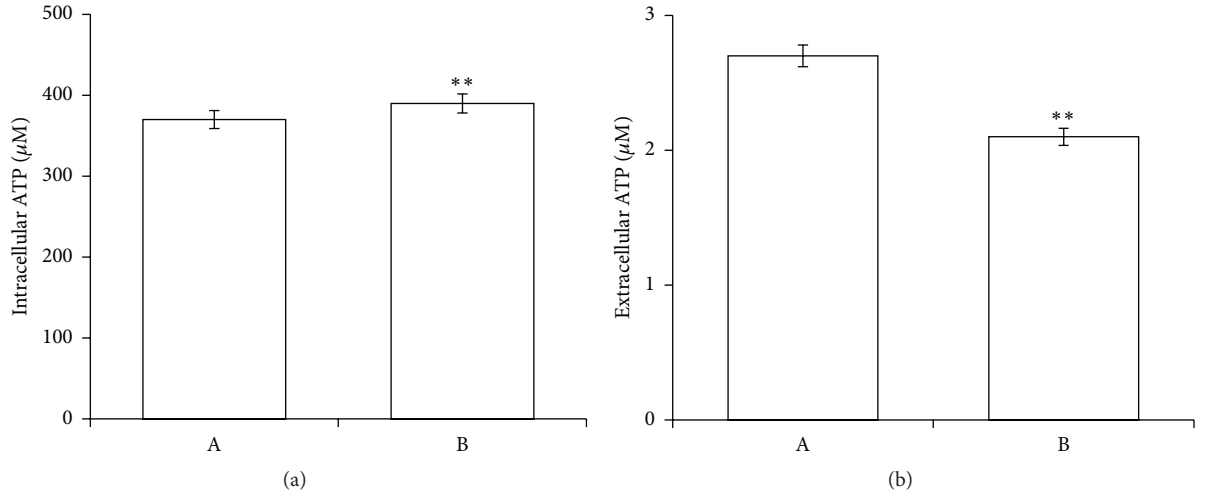

FIGURE 9: Effect of PID on the intracellular (a) and extracellular (b) ATP levels in RBCs. ATP concentrations were measured at the end of the incubation time of erythrocytes without (A) and with PID 100 μM (B). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ versus control.

cells experience a substantial need of reducing power in the form of NADPH for the biosynthesis of lipids and nucleotides required during proliferation. In this context, PID would interfere with cell proliferation not only by reducing NADPH and GSH availability, but also by positively modulating the functionality of pyruvate kinase M2 (PKM2) isoform expressed in cancer cells with low activity [66].

In particular, both the EMP enhancement, with the wider availability of fructose 1,6 biphosphate, and the cytosolic acidification induced by PID would activate the PKM2.

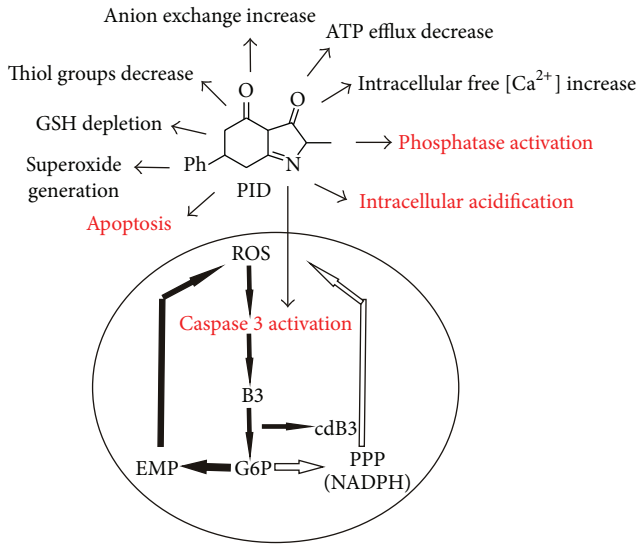

Figure 10: Effects of PID in RBCs and schematic representation of the "vicious circle" induced by PID. In red, the main antiproliferative conditions are highlighted.

Thus, the use of small molecule PKM2 activators may be an appropriate approach to interfere with cancer cell metabolism for therapeutic purposes.

In summary, this study contributes to highlighting the great potentiality of RBCs as versatile cellular model of study to predict the antiproliferative behaviour of selected heterocycles with different cellular localization. In particular, DPIT [3] is almost completely intercalated in the phospholipid bilayer, while PID crosses the RBC membrane. This different distribution leads to a series of complex metabolic responses that can be due to direct interactions/activations with cytosolic components and consequent increment of endogenous oxidative stress (i.e., PID) or to extracellular signals trigger that, on the whole, can culminate in the same increase of oxidative stress (i.e., DPIT). In particular, the effects of PID on RBCs, culminating in the caspase activation, would be represented in a "vicious circle" (Figure 10), in which the main antiproliferative conditions are highlighted.

Competing Interests

The authors declare that they have no competing interests regarding the contents of this paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9579868, 19 pages
http://dx.doi.org/10.1155/2016/9579868

Review Article

The Janus-Faced Role of Antioxidants in Cancer Cachexia: New Insights on the Established Concepts

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Received 10 March 2016; Revised 28 June 2016; Accepted 17 July 2016

Academic Editor: Alexandr V. Bazhin

Chronic inflammation and excessive loss of skeletal muscle usually occur during cancer cachexia, leading to functional impairment and delaying the cure of cancer. The release of cytokines by tumor promotes the formation of reactive oxygen species (ROS), which in turn regulate catabolic pathways involved in muscle atrophy. ROS also exert a dual role within tumor itself, as they can either promote proliferation and vascularization or induce senescence and apoptosis. Accordingly, previous studies that used antioxidants to modulate these ROS-dependent mechanisms, in cancer and cancer cachexia, have obtained contradictory results, hence the need to gather the main findings of these studies and draw global conclusions in order to stimulate more oriented research in this field. Based on the literature reviewed in this paper, it appears that antioxidant supplementation is (1) beneficial in cancer cachectic patients with antioxidant deficiencies, (2) most likely harmful in cancer patients with adequate antioxidant status (i.e., lung, gastrointestinal, head and neck, and esophageal), and (3) not recommended when undergoing radiotherapy. At the moment, measuring the blood levels of antioxidants may help to identify patients with systemic deficiencies. This approach is simple to realize but could not be a gold standard method for cachexia, as it does not necessarily reflect the redox state in other organs, like muscle.

1. Introduction

Approximately, 50% of patients with advanced stage of cancer experience cachexia and more than the third die following the loss of ~75% of skeletal muscle mass [1]. Cachexia is defined as a multifactorial syndrome characterized by a loss of more than 5% of total body weight mainly due to skeletal muscle wasting with or without depletion of adipose tissue [2]. Thus, the management of cancer cachexia is primordial to achieve a successful treatment. Pharmacological agents and single-nutritional interventions proposed to treat cachexia mainly resulted in an increase of fat mass but failed to effectively restore lean body mass [3, 4]. Indeed, muscle wasting is the component of cachexia that has the greatest negative impact on quality of life and anticancer treatment efficiency [2], hence the need to ameliorate our knowledge and understand the underpinning molecular mechanisms involved in cachexia-associated muscle catabolism.

Reactive oxygen species (ROS) are highly reactive, unstable, and short-lived molecules that play a crucial role in both health and disease [5]. Physiological amounts of ROS are produced endogenously (e.g., mitochondrial respiratory chain) and intervene in essential physiological mechanisms including phagocytosis, redox signaling, neurotransmission, proliferation, differentiation, and apoptosis [6–8]. Contrariwise, in pathological conditions, excessive ROS levels could lead to the development of oxidative stress (OS). OS is defined as a "disruption of the redox balance towards an increase in prooxidant over the capacity of antioxidants, leading to a perturbation of redox signaling and control and/or molecular damage" (i.e., lipids, protein, and DNA) [5, 9].

Several pieces of evidence suggest a key role for ROS in the development of muscle atrophy in response to the inflammatory profile related to cancer cachexia [10, 11]. Importantly, ROS also exert a double-faced role in tumor through triggering either growth/progression or death [8]. Accordingly, a number of clinical and preclinical studies of cancer and cancer cachexia have used antioxidants including vitamins E and C, β-carotene, α-lipoic acid, carbocysteine, and N-acetylcysteine, to antagonize or modulate these ROS-sensitive mechanisms. Unfortunately, the obtained results

were not always positives but sometimes without any significant effect or even deleterious [12–19]. Indeed, if the use of antioxidants appears to be complicated in cancer, it could be even more problematical in cancer cachexia given the intricate tissue crosstalk and the disruption of redox balance that takes place in many organs, including skeletal muscle, heart, liver, and blood [17, 20, 21]. In other words, high levels of ROS could be present at different sites, at the same time, and exert distinct roles in an organ-dependent manner. For example, the inhibition of ROS could be beneficial in skeletal muscle to reduce the magnitude of atrophy but deleterious within tumor as this may accelerate proliferation and growth [17, 22]. This multiorgan presence of ROS confers to cachexia an overelaborate nature and, thus, makes the intervention with antioxidants more perplexing.

Additionally, the self-prescription and uncontrolled use of supplements by patients may distort the conclusions regarding benefits or harms of antioxidant supplementation. Epidemiological studies have shown that more than 50% of patients increase their consumption of complements after diagnosis of cancer, without any medical prescription [23]. Antioxidant and nutritional supplements are used by cancer patients as they believe that these compounds feature a powerful anticancer activity [24]. Definitely, an adequate uptake of multivariate/multicolor fruits and vegetable is necessary for a healthy life-style and the world cancer research fund (WCRF) advise cancer patients to obtain antioxidants from food rather than supplements [25], whereas the random consumption of high-doses antioxidant complements is a real threat for cancer patients, as it can alter the efficacy of anticancer therapies and negatively influence tumor growth [26]. The use of antioxidants in cancer and cachexia has always been a polemical issue, hence the need to gather the main existing knowledge in an attempt to answer a number of essential questions and improve our understanding on this topic: how can the undifferentiated use of supplements by cancer patients impact tumor and anticancer treatment? Can some tumor types also benefit from antioxidants? How can we improve the use of such compounds? What is the factor that will provide eligibility for a cancer patient to undergo antioxidant supplementation?

2. Multiorgan Presence of Oxidative Stress Markers during Cancer Cachexia: Skeletal Muscle, Blood, Heart, and Liver

Clinical Studies. Oxidative damage markers were increased in the skeletal muscle of cachectic patients. Specifically, patients with lung cancer exhibited an increase in the levels of protein carbonyls in *vastus lateralis*, which correlated positively with muscle proteolysis [28]. The interesting study from Buck's team showed that lipid peroxidation adducts, malondialdehyde (MDA), were elevated within skeletal muscle (i.e., *vastus lateralis*) of patients with colon, lung, and esophageal cancer comparing to control subjects [29]. In addition to muscular OS, systemic OS seems to be exacerbated specifically after the onset of cachexia, since ROS production in the blood was greater in cachectic patients with lung cancer, comparing to noncachectic patients with lung cancer [30]. Mantovani and coworkers established a direct association between systemic OS and the performance status of cachectic patients. They found that the high blood levels of ROS were somehow associated with increased fatigue, decreased autonomy, and elevated concentrations of proinflammatory cytokines [31]. Liver biopsies from cachectic patients with esophageal, lung, and kidney carcinomas also revealed an increase in hepatic MDA-protein adducts [32]. Interestingly, the inflammatory profile associated with cachexia reduced the hepatic drug clearance in cancer patients *via* depressing the expression of cytochrome P450 (CYP) in liver, namely, CYP3A [33, 34]. This could prolong the blood exposures of drugs and increase toxicity risk in patients undergoing chemotherapy. Furthermore, CYP3A is involved in the metabolism of several opioid analgesics used to alleviate cachexia symptoms; thus the decrease in CYP3A expression and activity could also affect the management of pain in cancer cachectic patients [33, 34].

Animal Studies. OS was also reported in skeletal muscle and other tissues of cachectic animals. For example, protein carbonylation and lipid peroxidation adducts, namely, 4-Hydroxynonenal (4-HNE) and MDA, were increased in the *gastrocnemius* (Gas) muscle of rats bearing Yoshida AH-130 hepatoma tumor [35]. In our own laboratory, we have shown that implantation of colon 26 (C26) cells into BALB/c mice induced cachexia and skeletal muscle atrophy. Cachectic C26 mice exhibited a net augmentation in protein carbonyls and 4-HNE content within plasma, without any change in skeletal muscle. The absence of muscular oxidative damage in our model could be attributed to the upregulation of catalase expression, exclusively, in atrophied muscles [17]. Other experimental studies have also shown that mice bearing Walker 256 and MAC13/16 tumors developed cardiac cachexia in response to DNA and/or protein oxidative damage in heart tissues [20, 36]. Additionally, mice bearing C26 tumor exhibited an upregulation in gene-specific inflammation within heart and manifested a reduction in cardiomyocytes diameter, loss of ventricular mass, and systolic dysfunction [37–39]. Indeed, the treatment of primary rat cardiomyocytes with the conditioned milieu of C26 cells induced atrophy, increased mitochondrial stress, and triggered an aberrant lipid oxidation metabolism [39]. These data suggest that tumor-borne factors promote cardiac dysfunction in cachexia. Besides heart atrophy, cachexia was able to suppress the expression of CYP in liver of mice [40] and increase ROS production ~12-fold in liver of cancer bearing rats [21]. Therefore, tumor-derived factors are mainly responsible for the deregulation of body redox homeostasis and the development of OS that might lead to multiorgan failure and enhance cachexia progression (Figure 1). As skeletal muscle wasting is a key feature of cancer cachexia, hereafter, we will focus and describe main ROS-dependent mechanisms involved in muscle proteolysis and the interplay between tumor and muscle.

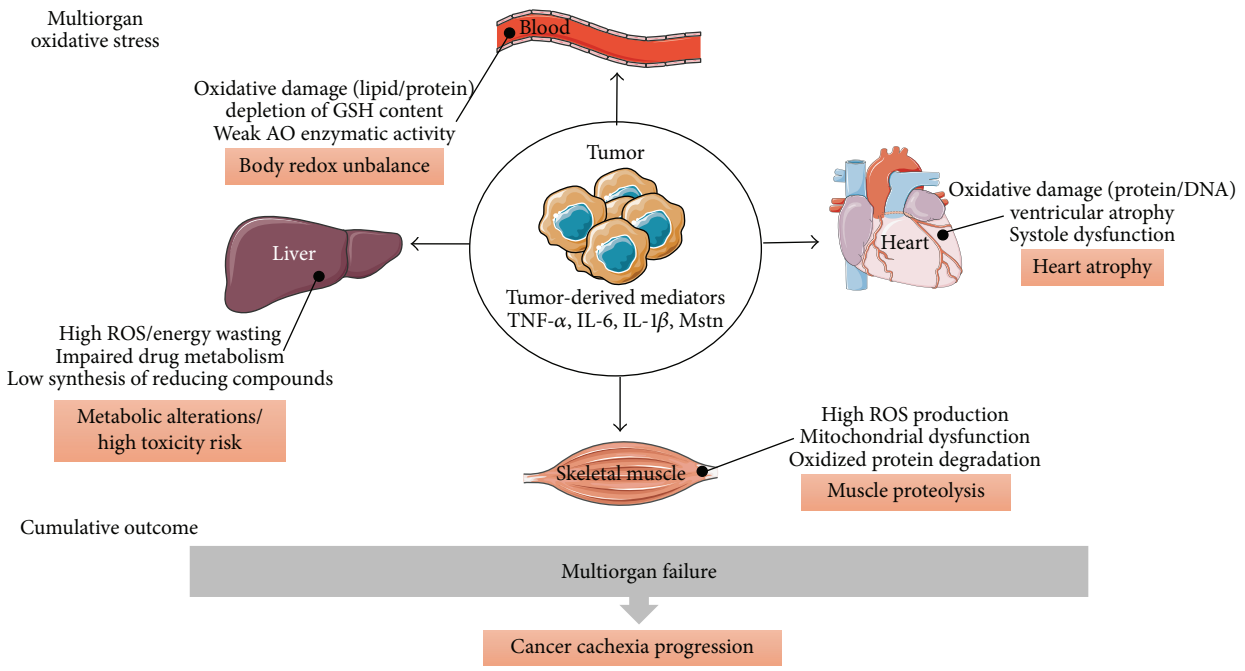

Figure 1: The central role of tumor in the development of oxidative stress at multiple organs during cachexia. Tumor is the main responsible factor for the development of OS at different organs and the consecutive disruption of their vital functions. Indeed, chemicals released by tumor in the systemic circulation can reach multiple destinations like heart, muscle, and liver. For example, TNF-α and IL-6 can induce anorexia, leading to inadequate synthesis of reducing compounds like NADPH in the liver. Additionally, IL-6, TNF-α, and myostatin (Mstn) upregulate the activity of ROS-producing enzymes within heart/skeletal muscles, leading to the activation of several catabolic pathways and muscle proteolysis. As a direct result, heart/skeletal muscles are atrophied, oxidative injuries accumulated, and antioxidant (AO) defense becomes inefficient, giving way to multiorgan failure and cancer cachexia evolution.

3. ROS Production and Inflammation: Causality Link and Principal Mechanisms

3.1. Tumor-Derived Chemicals

Clinical Studies. Proinflammatory cytokines, transforming growth factor-beta (TGF-β) family ligands, and other tumor-specific mediators like proteolysis-inducing factor (PIF) are expressed and released continuously by tumor cells [41]. Once in bloodstream, these mediators can easily reach skeletal/cardiac muscles and promote ROS formation by binding to their cognate receptors expressed on the surface of muscle cells [42]. Basically, ROS promote muscle wasting and cachexia progression through the activation of three main catabolic pathways: ubiquitin proteasome system (UPS), autophagy lysosome pathway, and calcium-dependent calpain pathway. Elevated levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and PIF were reported in biological fluids (e.g., blood and urine) of patients experiencing cachexia [28, 29, 31, 43]. In pancreatic cancer patients, systemic inflammation was correlated with the activation of proteasome system in skeletal muscle [44]. Interestingly, gastric cancer patients with no weight loss exhibited an increase in calpain activity in the *rectus abdominis* muscle, without any change in the expression of key components of the UPS, *MuRF-1,* and *MAFbx* [45]. On the other hand, proteasome activity was significantly higher within *rectus abdominis* of weight-losing patients with advanced stage of gastric cancer [46]. These findings may emphasize the fact that calpains are activated earlier during cachexia related to gastric cancer, before substantial weight loss and hypercatabolism of skeletal muscle by the UPS. In other cancers, such as esophageal cancer, muscle proteolysis seems to be dependent on the activities of lysosomal proteases, cathepsins B and L, indicating a possible involvement of autophagy in the pathogenesis of muscle wasting clinically [47]. Together, these data may suggest that the activation of a specific catabolic pathway depends on the type of cancer and, therefore, the nature of circulating humoral factors. For example, excessive skeletal muscle loss and cachexia related-death culminate in patients with colorectal, pancreatic, and lung cancer, whereas those with breast, sarcomas, and non-Hodgkin's lymphoma are usually spared [48].

Animal and Cell Culture Studies. The presence of high ROS levels within muscle cells alters the function of numerous organelles, which in turn may induce muscle dysfunction and foster the degradation process of sarcomeric proteins [49]. For example, hydrogen peroxide (H_2O_2) induces endoplasmic reticulum stress leading to myoplasmic calcium (Ca^{2+}) accumulation and, therefore, the activation of calpains [50, 51]. Calpains promote the disintegration of sarcomere structure and liberation of actin/myosin filaments for the

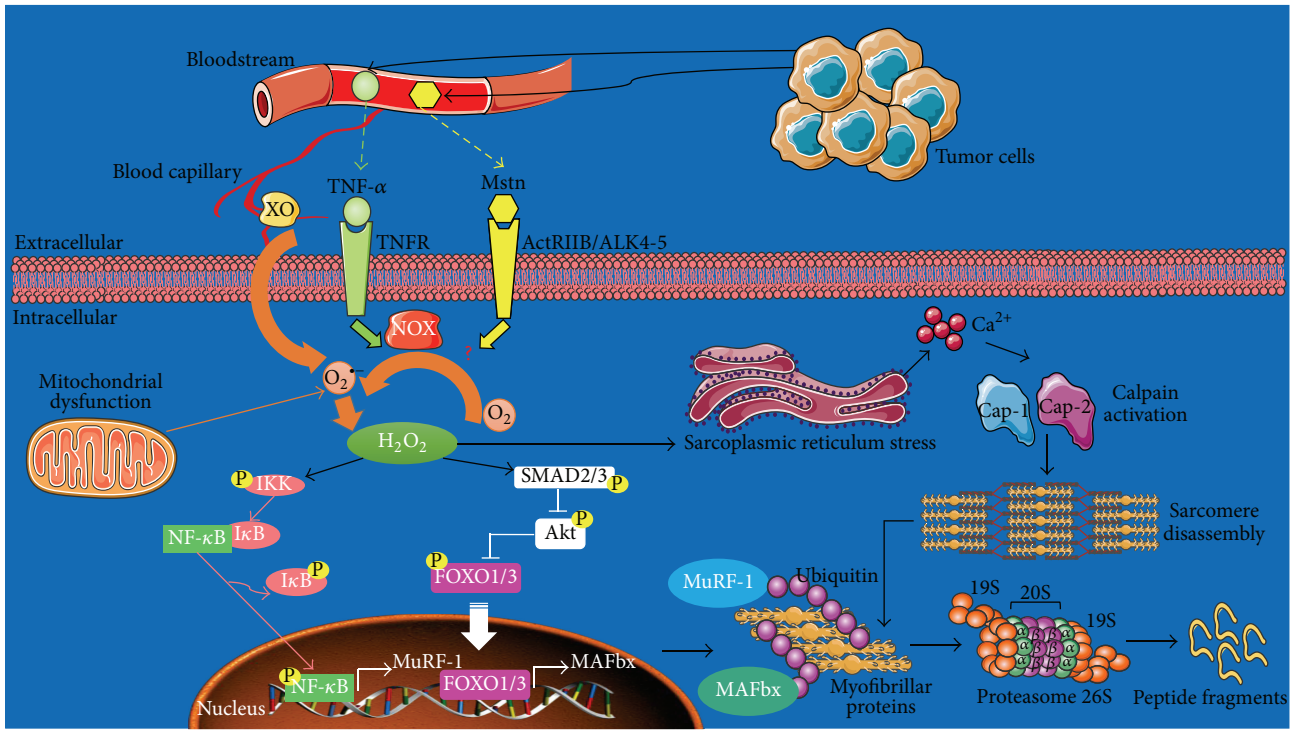

FIGURE 2: Role of ROS as a second messenger in the activation of proteolysis pathways. Tumor cells produce great amounts of proinflammatory cytokines and TGF-β family ligands, such as TNF-α and Mstn, respectively. Once in bloodstream, these mediators can easily reach skeletal muscle and activate several catabolic pathways, by signaling through their specific receptors. TNF-α induces the activation of NOX found in muscle fibers. The elevated activity of NOX and XO (XO is usually located within blood capillaries irrigating muscle) during cachexia is responsible for the great production of anion superoxide ($O_2^{\bullet-}$) molecules, which are rapidly converted into hydrogen peroxide (H_2O_2). Accumulation of H_2O_2 within muscle fibers induces sarcoplasmic reticulum stress and the subsequent massive release of calcium (Ca^{2+}) ions. The increase of intracellular Ca^{2+} concentrations activates calpains 1 and 2 (Cap-1 and Cap-2), which in turn promote sarcomere disintegration and myofibrillar proteins liberation. H_2O_2 can activate IkB kinase (IKK) or SMAD3, leading to the phosphorylation of IkB and the dissociation of the NF-kB/IkB complex. Subsequently, NF-κB is released and ready to translocate into the nucleus. Additionally, P-SMAD2/3 transducers remove the sustained inhibitory phosphorylation of P-FOXO1/3 exerted by Akt and, therefore, allow its nuclear accumulation. Upon their entry into the nucleus, P-NF-κB and FOXO1/3 promote the transcriptional activation of MURF-1 and MAFbx, respectively. Then, MURF-1 and MAFbx tagged myofibrillar proteins with polyubiquitin chains to undergo proteolytic processing by the proteasome core (adapted from [27]).

proteasome machinery [49]. In parallel, ROS mobilize various transcriptional factors directly involved in the regulation of genes related to catabolic pathways. A previous study demonstrated that nuclear factor-κB (NF-κB) was rapidly activated by H_2O_2, following treatment of C2C12 muscle cells with TNF-α [10]. We have also shown that circulating levels of TNF-α were increased in cachectic mice bearing colon tumor and coincided with a greater phosphorylation of the NF-κB (p65) subunit, within atrophied muscles [17]. The nuclear accumulation of NF-κB promotes the transcriptional upregulation of muscle-specific E3 ubiquitin-ligases, *MuRF-1* and *MAFbx*, which in turn tag myofibrillar proteins (i.e., myosin) with polyubiquitin chains for proteasome processing [52]. NF-κB also induces the expression of proteasome subunits and proinflammatory cytokines, like IL-6, thereby maintaining a vicious circle [53, 54]. In the same way, IL-6 was described as a potent activator of signal transducer and activator of transcription 3 (STAT3), which controls the activation of UPS-dependent elements [55] and both expression and activity of cathepsins B and L in the atrophied muscles [11]. Accordingly, the blockade of IL-6 with a specific antibody attenuated cachexia severity and muscle wasting in C26 mice [11]. High levels of Forkhead box (FOXO) were also reported in muscles of cachectic animals [56]. Thus, emerging pieces of evidence suggest a possible role for ROS in controlling the transcriptional activity of FOXO [57], which is a master regulator of a plethora of genes related to the UPS and autophagy mechanisms such as autophagosome biosynthesis and autophagosome-lysosome fusion [58]. Figure 2 illustrates the role of ROS as a second messenger in the activation of main catabolic pathways within skeletal muscle, in response to *bona fide* tumor cytokines.

3.2. Eicosanoids

Clinical Studies. Lipoxygenase (LOX) and cyclooxygenase (COX) are two enzymes producing potent inflammatory mediators called eicosanoids. Brain, skeletal muscle, and some tumor types express both enzymes and specific receptors for eicosanoids [59]. Three isoforms are identified for

LOX (5-LOX, 12-LOX, and 15-LOX) and two for COX (COX-1 and COX-2). Arachidonic acid (AA) constitutes the main substrate for LOXs, to produce leukotriene (LT) and hydroxyeicosatetraenoic (HETE) acid, and for COX in the synthesis process of prostaglandin (PG) and thromboxane (TAX) [59]. Clinically, six-week selective inhibition of COX-2, using celecoxib, reduced the severity of cachexia symptoms in lung cancer patients through improving muscle strength and lowering the circulating levels of C-reactive protein (CRP) (marker of systemic inflammation) [60]. Treatment with celecoxib, during four months, was also effective in attenuating the blood levels of TNF-α, decreasing fatigue, and increasing lean body mass in patients with ovary, pancreas, and colorectal cancer [61]. Similar findings were obtained from patients with head and neck cancer treated with celecoxib for three weeks [62]. These clinical results suggest a potential role for COX-2 in promoting chronic inflammation observed in cancer cachexia and the related muscle wasting. Nonetheless, there is a lack of information concerning the regulation of LOX in clinical cancer cachexia.

Animal and Cell Culture Studies. In experimental cancer cachexia, the inhibition of 5-LOX using CV-6504 attenuated tumor growth and cachexia progression in animals bearing MAC16 and MAC26 adenocarcinoma [63, 64]. Additionally, the inhibition of COX-1/2 using indomethacin or COX-2 with NS 398 rescued muscle wasting related to Lewis lung carcinoma (LLC) or C26 tumor but had no effect on muscle loss in mice bearing B16 melanoma [65, 66]. Importantly, the preservation of muscle mass was due to the regression of tumor growth and reduction in circulating eicosanoids and IL-6 amounts as well as the decrease in TNF-α receptor-1 levels within Gas muscles [65–67]. These findings indicate that the crosstalk between tumor and skeletal muscle and the resulting catabolic response depend largely on LOX/COX metabolites and cytokines. Importantly, these eicosanoids could mediate the catabolic actions of tumor-derived cytokines through activating a number of ROS-producing enzymes and increasing ROS generation [68, 69]. For example, in response to specific tumor factors, high levels of 15-HETE could be produced to enhance ROS production and protein degradation within muscle cells [69]. Therefore, we suppose that "cytokines-eicosanoids-ROS-muscle catabolism" is the main axis through which tumor induces muscle loss during cachexia.

4. Main Sources of ROS in Cancer Cachexia

4.1. Elevated Activity of ROS-Producing Enzymes

4.1.1. Xanthine Oxidase

Clinical Studies. In normal conditions, the highest levels of xanthine oxidoreductase (XOR) activity are present in intestine of mammals, contrary to muscles tissues in which XOR activity is very low [70]. XOR exists in two interconvertible forms that are xanthine dehydrogenase (XDH) and xanthine oxidase (XO). In several pathological states, the presence of proinflammatory cytokines promotes the cleavage of XDH to XO, which instead uses molecular oxygen to catalyze the hydroxylation of hypoxanthine to xanthine and, then, to uric acid, producing ROS, mainly anion superoxide ($O_2^{\bullet-}$) and H_2O_2 [71]. The role of XO was mainly addressed in cancer patients, regardless of the stage of cachexia. Herein, we will describe a number of these studies, in an attempt to elaborate a hypothesis about the eventual role of XO in cancer cachexia. Studies in humans demonstrated an increase in blood XO activity in patients with non-small-cell lung carcinoma (NSCLC), small-cell lung carcinoma (SCLC), head and neck carcinoma, and liver cancer compared to control patients [72–74]. The activity of XO was in most cases positively correlated with prooxidant parameters in blood samples (i.e., lipid peroxidation adducts) [72, 74]. An elevated activity of XO was also noted in the plasma of patients with acute lymphoblastic lymphoma, while patients with cervix cancer exhibited a low activity of XO [75]. However, there is a lack of information concerning the modulation of XO activity in the skeletal muscle of cancer patients. Based on clinical data, it appears that the activity of XO in blood is most likely elevated in cancer patients and, therefore, its inhibition could be beneficial. Accordingly, accumulating evidences from animal studies globally support an involvement of XO in the pathophysiology of cancer cachexia. Thus, the activity of XO is expected to increase in cachectic cancer patients, but clinical studies are still needed to confirm such hypothesis.

Animal Studies. In the experimental models of cancer cachexia, rats bearing Yoshida tumor and mice bearing MAC16 adenocarcinoma, the activity of XO was elevated in skeletal and/or cardiac muscles and correlated with an increase in muscle oxidative damage [20, 76–78]. Although XO is not usually present at high levels within skeletal muscle, the hyperactivation of XO during cachexia could be explained by an increase in the cleavage of XDH to XO [76]. The small number of studies that addressed the role of XO in cachexia-induced muscle wasting demonstrated that targeting XO with selective inhibitors such as allopurinol (4 and 40 mg/kg/day), oxypurinol (4 and 40 mg/kg/day), and febuxostat (5 mg/kg/day) can reduce body weight loss and skeletal muscle/heart atrophy [76–78]. The molecular mechanisms behind these beneficial effects of XO inhibition are mainly (1) attenuation of oxidative damage within skeletal muscle, (2) inhibition of DNA binding potential of transcription factors like NF-κB and STAT-3, (3) reduction of proinflammatory cytokines expression, (4) decrease in the expression of key components of the UPS (e.g., ubiquitin, MuRF-1), and (5) reinforcement of protein synthesis pathways (e.g., Akt activation) [76–78]. Preliminary results from our laboratory indicate that treatment of C26 tumor-bearing mice with allopurinol (50 mg/kg/day) partially prevented the decrease in *extensor digitorum longus* (EDL) muscle fiber diameter but failed to improve total body and skeletal muscle weight loss (Table 1). This could be attributed to the fact that protein carbonyls and 4-HNE content, although present in plasma, were absent in skeletal muscle, while in the study of Springer et al., showing improvement of muscle mass after allopurinol administration, the content of protein

Table 1: Impact of allopurinol on cachexia symptoms in C26 mice. Balb/C mice subcutaneously inoculated with 1×10^6 C26 cells have received daily dose of allopurinol (50 mg/kg/day) or vehicle (PBS). Mice weight was daily monitored and skeletal muscles were weighted at the end of the protocol. Fiber diameter was determined from at least 100–150 fibers per muscle histological section, stained with the Gomori method. Data are mean ± SEM (n = 8/group).

	Control	C26	C26-allo
Initial body weight (g)	23.6 ± 0.6	23.4 ± 0.6	23.9 ± 0.7
Final body weight (g)	25.9 ± 0.5	21.5 ± 2.1	20.1 ± 1.8
ΔBody weight (g)	2.3 ± 0.5	−2.2 ± 2.05[a]	−3.8 ± 1.3[a]
Soleus weight (mg)	7.1 ± 1.7	6.4 ± 2.3	7 ± 3.3
Gas weight (mg)	128.1 ± 14.4	94.5 ± 15.1[a]	91.5 ± 22.7[a]
EDL weight (mg)	10.7 ± 2.3	8 ± 1.8[a]	8.2 ± 1.5[a]
EDL fiber diameter (μm)	41.62 ± 2.4	29.8 ± 5.7[a]	36.8 ± 5.2[b]

[a] $P < 0.001$ versus control; [b] $P < 0.01$ versus C26; Gas: *Gastrocnemius*; and EDL: *extensor digitorum longus*.

carbonyls was greater within wasted muscles and significantly decreased in response to allopurinol [76]. Additionally, allopurinol failed to attenuate systemic oxidative damage in C26 mice. This may indicate that XO is not a primary actor in the pathogenesis of muscle wasting related to C26 tumor.

4.1.2. Nicotinamide Adenine Dinucleotide Phosphate Oxidase

Clinical Studies. The family of nicotinamide adenine dinucleotide phosphate oxidase (NOX) produces both $O_2^{\bullet-}$ and H_2O_2[79]. Seven isoforms have been identified to produce ROS, among which NOX-4 produces H_2O_2 and NOX-1, NOX-2, and NOX-5 generate $O_2^{\bullet-}$ [79]. In conditions evoking chronic inflammation, which is the case of cachexia, high amounts of ROS originating from NOX could negatively influence gastrointestinal and pancreatic cancer development [80]. Clinically, the expression of NOX-1 and NOX-4 in tumor was associated with poor survival and cancer relapse [81, 82]. Another isoform, NOX-5, was also found to be overexpressed in numerous cancers, including colon, melanoma, breast, lung, and prostate cancer [80]. However, the role of NOX in cancer and cancer cachexia has not been addressed in depth clinically and further studies are needed to establish its exact role. At the moment, it seems that the expression of NOX within tumor is associated with cancer progression [80].

Animal and Cell Culture Studies. TNF-α, IFN-γ, PIF, and Angiotensin-II (Ang-II) are known to induce ROS production *via* the activation of NOX [83, 84]. In a model of Ang-II-infused mice, the high formation of $O_2^{\bullet-}$ levels within muscles upregulated the expression of E3-ligases MuRF-1/MAFbx and promoted proteasome-mediated proteolysis [83]. This elevated production of $O_2^{\bullet-}$ was NOX-dependent, since its blockade with a specific inhibitor, apocynin, partially prevented atrophy. Contrariwise, it is thought that the enhanced $O_2^{\bullet-}$ formation within skeletal muscle of cachectic mice bearing MAC16 tumor was due to an aberrant antioxidant response rather than an increase in NOX activity [85]. PIF was able to promote phospholipase A2-catalyzed release of AA from membrane phospholipids. The conversion of AA into 15-HETE, by 15-LOX, promoted NOX-induced $O_2^{\bullet-}$ production and the subsequent activation of NF-κB/UPS proteolysis pathway in muscle cells [69]. In addition to skeletal muscle, LOX/NOX signaling is one of the prosurvival mechanisms that makes pancreatic cancer cells unresponsive to anticancer treatments [86]. Since NOX controls the activation of various downstream kinases that play an essential role in proliferation, differentiation, and inflammation, the silencing of NOX isoforms, especially NOX-4, could provide a particular therapeutic interest to limit cancer cells proliferation and reduce the magnitude of muscle degradation.

4.1.3. Nitric Oxide Synthase

Clinical Studies. Nitric oxide (NO) is a free radical produced enzymatically by NO synthase (NOS) from L-arginine. NOS exists in three different isoforms: Type I NOS and Type III NOS (eNOS), expressed constitutively in the skeletal muscle, and Type II NOS also called inducible NOS (iNOS) expressed exclusively in the presence of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1 [87]. At high concentration, NO can induce nitrosative stress through reacting with $O_2^{\bullet-}$ and, subsequently, producing elevated levels of peroxynitrite molecules extremely injurious for muscle [5]. Nitrotyrosine is usually used as a biomarker to evaluate the level of nitrosative damage. Today, it is admitted that the arginine/NO metabolism is altered in cachectic patients and responsible for the inhibition of protein synthesis and activation of proteolysis [88]. High NO levels were found in plasma of patients with gastric cancer comparing to those without cancer [89]. Cachectic patients with advanced stages of cancer presented a greater NO production, nitrotyrosine content, and iNOS expression in skeletal muscle tissues, comparing to noncachectic subjects [29, 87]. Importantly, iNOS was also found to be expressed in tumor tissues of patients and its expression correlated positively with tumor size and aggressiveness, especially in breast and colorectal cancer [90].

Animal and Cell Culture Studies. In cachectic nude mice overexpressing TNF-α gene, the NOS system was activated and responsible for the disruption of D-Jun/myogenin-complex binding to the myosin creatinine phosphokinase enhancer (MCK-E) box, leading to muscle atrophy and

dedifferentiation [91]. The inhibition of NOS, by nitro-L-arginine, prevented weight loss and muscle wasting in TNF-α-treated animals [91]. Apoptosis is one of the mechanisms that could be involved in muscle atrophy. Caspase-3, jointly with calpains, mediates the dissociation of actinomyosin complex, making myofilaments susceptible to UPS degradation [49]. Interestingly, a link between iNOS and apoptosis activation has been suggested, since the administration of IL-15 to cachectic rats inhibited apoptosis by disturbing TNF-α signaling and the resulting NO formation [92]. In C2C12 cells, TNF-α and IFN-γ were able to induce the activation of NF-κB and its downstream target iNOS [93]. The activation of TNF-α/NF-κB/iNOS pathway was efficient to promote the degeneration of muscle *via* stimulating the loss of proteins playing a key role in muscle cell proliferation and differentiation such as MyoD [93]. These compelling evidences indicate that selective inhibition of iNOS could decelerate cachexia progression in cancer.

4.2. Mitochondrial Dysfunction. A scarce number of preclinical studies have addressed the mitochondrial events that occur within skeletal muscle during cancer cachexia but data from humans are still lacking. Mitochondrial dysfunction and altered mitochondrial plasticity are a primary source of ROS generation in cachexia. ROS exert direct deleterious effects on mitochondrial respiratory chain (MRC) complexes (i.e., complexes I, II, and IV) by decreasing their activities in skeletal and respiratory muscles of cachectic mice [94]. Thus, it makes sense that ROS-mediated MRC dysfunction could lead to impaired oxidative phosphorylation and low adenosine triphosphate (ATP) synthesis. In numerous animal models of cachexia related muscle wasting, skeletal mass degradation was associated with a decrease in respiratory chain activity and low ability of wasted muscles to synthetize the required ATP [95, 96]. Indeed, treatment of C2C12 muscle cells with LLC conditioned culture medium (rich in proinflammatory mediators) increased ROS production and reduced ATP production [97]. These disruptions in respiratory chain function were mainly due to mitochondrial loss (i.e., mitophagy), structural abnormalities (i.e., giant mitochondria), and increased uncoupling proteins (UCPs) expression, namely, UCP2 and UCP3 [98, 99]. As depicted in Figure 3, a weak ATP production leads to a low mitochondrial transmembrane potential [100], allowing mitochondria to generate excessive amounts of ROS potentially damaging for mitochondria membrane and muscle. Thus, there is a ROS-ATP-ROS loop during cachexia. ROS primarily produced in response to inflammatory stimuli disturb the MRC function within muscle, leading to a decreased ATP formation [97]. This poor ATP level is a favorable condition for high mitochondrial ROS production [100], thereby maintaining the vicious circle. The mitochondrial energetic inefficiency and the subsequent accumulation of oxidative insults may impede the capacity of muscle to generate sufficient force and ensure basic physical needs [101]. This ROS-dependent mechanism observed in skeletal, cardiac, and respiratory muscles may in part explain the increased fatigue and decreased autonomy observed in cachectic individuals with advanced stages of cancer.

4.3. Defective Antioxidant Responses

Clinical Studies. In addition to the above-mentioned sources of ROS, the loss of antioxidant counterbalance and control can exacerbate OS in cancer cachexia. At the systemic level, SOD activity was upregulated in patients, with stage II to stage IV cancer, presenting a good performance status, while SOD activity decreased along with GPx activity in cachectic patients with compromised physical performance at stage IV [31], indicating that high grade cancer and poor muscle strength are, most likely, associated with a weak enzymatic antioxidant activity. Furthermore, patients bearing breast or colon cancer displayed a low blood level of reduced glutathione (GSH) [102]. The decrease in GSH content may be due to a decrease in the available substrates needed for GSH synthesis. In fact, glucose plays a pivotal role in the synthesis of compounds with high reducing potential, like NADPH, through the pentose phosphate pathway. NADPH is required for (1) the reduction of GSH disulphide (GSSG) to GSH, by the GSH reductase, and (2) formation of active catalase tetramers [103]. The perturbations in glucose metabolism and reduced nutrients supply, due to symptoms such as anorexia and vomiting, can lead to an inadequate synthesis of reducing compounds and, therefore, may explain the GSH deficiency observed in cachectic individuals [104].

Animal and Cell Culture Studies. Treatment of C2C12 cells with TNF-α caused a net decrease in GSH content, which coincided with elevated ROS generation and atrophy development [105]. In line with these *in vitro* findings, both expression and activity of SOD and GPx decreased in the skeletal/cardiac muscles of cachectic mice [20, 85, 106]. On the other hand, other experimental studies found that the expression of SOD was upregulated within atrophied skeletal muscles [28, 76]. We have reported an increase in catalase expression within skeletal muscle of cachectic mice without any change in CuZnSOD and MnSOD expression [17]. Nonetheless, studies that denoted an increase in SOD activity have also demonstrated an increase in OS profiles, suggesting that SOD activation was inefficient and insufficient to antagonize muscular and systemic OS. An accumulation of high H_2O_2 rates due to the elevated SOD activity might explain this paradox. However, data available from the literature strongly suggest that the decrease of muscle and blood GSH content, GSH/GSSG ratio, and GPx activity occur during cancer cachexia related muscle wasting. In addition to the involvement of ROS in the pathophysiology of muscle wasting, these species directly regulate the growth/death balance within tumor itself and several ROS-dependent mechanisms have been unveiled (see Section 6).

5. The Dual Role of ROS in Tumor

Clinical Studies. The role of ROS in cancer has been previously discussed in detail [107, 108]. Contrary to skeletal muscle in which lessening oxidative damage is relatively advantageous during cachexia, the reduction of tumor OS could be deleterious in some cases. ROS play a dual role within tumor; on the one hand they have the ability to promote tumorigenesis

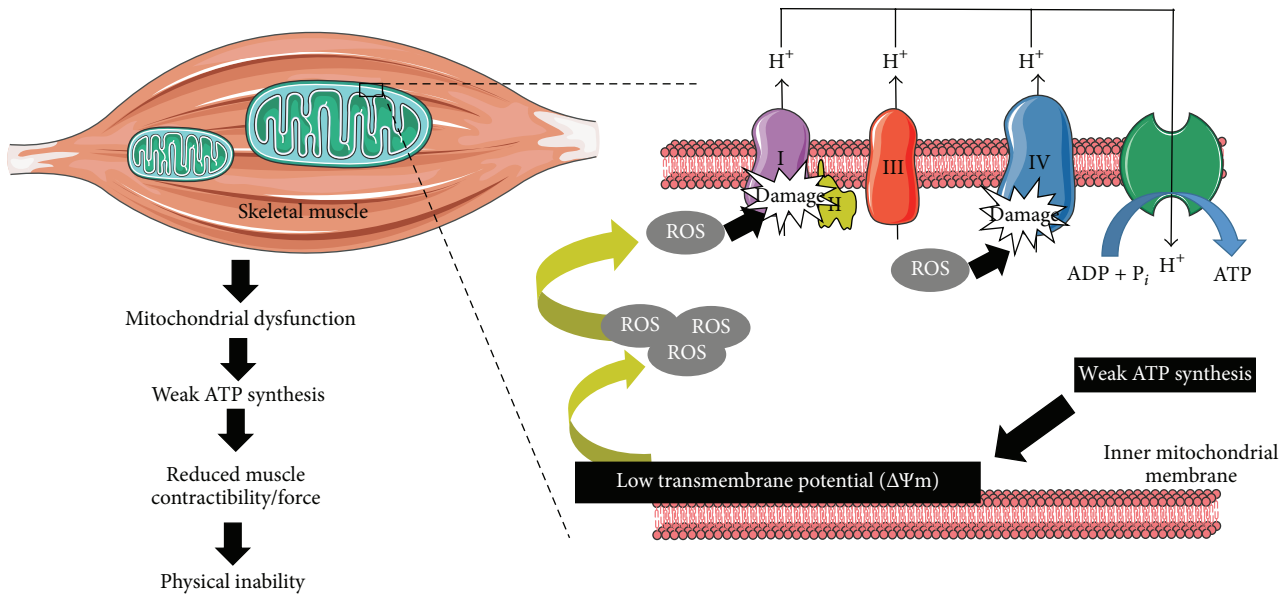

Figure 3: Mitochondrial dysfunction in wasted muscles. High ROS amounts present within atrophied muscles impair mitochondrial ATP synthesis by causing direct oxidative damage in the electron transport chain. This weak ATP production leads to a low mitochondrial transmembrane potential, allowing mitochondria to produce very excessive rates of ROS, thereby maintaining the vicious circle. All these events contribute to muscle wasting development through impairing muscle contractibility and ability to generate force.

and vascularization [109]. On the other hand they can induce DNA damage, cell cycle arrest, and apoptosis [8]. This double-faced role of ROS was underscored clinically. Accordingly, high levels of ROS were detected in human hepatocellular cancerous tissues comparing to normal adjacent tissues [110]. It is though that ROS accumulation could promote cancer progression *via* the activation of several transcriptional factors, including FOXO6, regulating the expression of cell cycle genes (i.e., p27 and cyclin-D1) [110]. Thus, in this case, the inhibition of ROS could be beneficial to slow cancer growth. But ROS can also generate intracellular signals that stimulate cell death, and new anticancer targeted therapies using encapsulated nanoparticles (i.e., HSP90 inhibitor) mainly rely on the generation of excessive ROS amounts to promote apoptosis and improve cancer care [111]. Therefore, *can some tumor types also benefit from antioxidants?* Indeed, the impact of antioxidant supplementation on both tumor progression and regression was mainly addressed in animals.

Animal Studies. Accumulating evidence from high-quality studies indicates that antioxidants could be detrimental in cancer bearing mice. Piskounova et al. elegantly demonstrated that high ROS levels protected against melanoma metastasis in NSG mice, since metastatic cells presented a lower ROS generation comparing to subcutaneous non-metastatic tumor [112]. Similar findings were obtained by Le Gal et al. showing that administration of antioxidants enhanced the invasive potential of melanoma tumors without affecting proliferation [113]. Thus, in addition to the modulation of cell cycle, ROS control tumor behavior through the regulation of cytoskeletal proteins involved in cell migration and invasion [113]. We have recently shown that the reduction of tumor OS in cachectic mice bearing C26 colon cancer accelerated proliferation [17]. Contrariwise, in rats bearing AT-1 prostate cancer, the inhibition of OS decreased tumor oxidative damage and proliferation [114], indicating that the reduction of OS could either enhance or slow tumor proliferation and progression depending on tumor type and localization. In other words, the redox state of tumor is an important factor that could swing the balance of a given antioxidant treatment towards the beneficial or harmful side. Thereafter, in some cases the inappropriate use of antioxidants could promote tumor growth through decreasing ROS production and oxidative damage. A direct consequence of the enhanced tumor growth is an increase in the circulating levels of tumor-derived mediators and the subsequent cachexia development.

6. Antioxidant Supplementation in Cancer Cachexia: Impact and Molecular Mechanisms

6.1. Antioxidant Vitamins and Carotenoids

Clinical Studies. No previous studies have addressed the role of individual vitamins in cancer cachexia. Antioxidants were usually given as a mixture containing vitamins, polyphenols, and other antioxidant compounds [13, 14]. Most intervention studies with antioxidant vitamins performed on cancer patients did not explore the concept of cachexia or take into account cachexia staging criteria to select patients. However, as mentioned above, tumor occupies a central role in the development of cachexia; thus, in a first step it could be helpful to draw a global view about the impact of vitamins on cancer itself with the aim of better using these products in

cancer cachexia. Data available from clinical studies suggest a lack of convincing evidence concerning the beneficial effects of vitamin supplementation in cancer patients [115]. The systematic review and meta-analysis of Bjelakovic et al. incorporated the results of 14 randomized trials and concluded that high-doses of vitamin A/E and β-carotene were associated with increased mortality in patients with gastrointestinal cancer [18, 116]. Accordingly, the meta-analysis from Pais and Dumitraşcu indicated that the combination of β-carotene with vitamin E could increase mortality in patients with colorectal cancer [117]. Interestingly, this increase in mortality seems to be more pronounced when doses of vitamin E exceeded 134 mg/day [118]. Thus, if antioxidant vitamins are problematical in some cases, *how should we improve the use of such compounds?* In other words, *what is the factor that will provide eligibility for a cancer patient to undergo antioxidant supplementation?* The response seems to be provided by the randomized double-blinded trial "SUVIMAX." At the baseline, healthy men enrolled in the study exhibited a low blood antioxidant status compared to healthy women, because of the reduced intake of fruits/vegetables often observed in men's alimentary habits [119]. After eight years of daily supplementation with complements including vitamin C/E and β-carotene at nutritional doses, men presented a reduced risk of 31% to prostate cancer, while women with adequate antioxidant status at the baseline developed an increased risk of 67% to skin cancer [119], indicating that only individuals with particular antioxidant deficiencies will benefit from supplementation in terms of cancer prevention. This conclusion, although obtained in disease-free subjects, could be logically transposable to cancer cachectic patients.

The small number of intervention studies with antioxidants conducted on cachectic patients with head and neck, ovary, colorectal, lung, and breast cancer supports the evidence that vitamins in combination with other antioxidants could be beneficial in patients with weak blood antioxidant activity and high ROS levels [120]. Intriguingly, a previous study has shown that patients with lung cancer exhibited low blood levels of vitamin E comparing to controls, but the depletion of vitamin E was more pronounced in cachectic patients [30]. This may indicate that even in the same type of cancer the doses of vitamins must be adapted taking into account the presence or absence of cachexia. Recently, the French speaking society of clinical nutrition and metabolism (SFNEP) has discouraged the use of α-tocopherol and β-carotene for patients with esophageal and head and neck cancer without diagnosed deficiency [121]. The SFNEP has also stressed out the negative impact of a high-dose and long-term antioxidant vitamins administration on the effectiveness of radio/chemotherapy [121]. Accordingly, supplementation with vitamin E and β-carotene increased cancer recurrence and overall mortality in head and neck cancer patients undergoing radiotherapy [122]. According to the review of Harvie it seems that the association of antioxidant vitamins with radiotherapy reduces its anticancer potential [24]. However, there is a lack of evidence concerning the combination of vitamins with chemotherapy. At the moment, the best way to provide an effective nutritional support for cachectic cancer patients is to determine and adapt vitamins doses on a patient-by-patient basis. The supplementation must target cachectic patients exhibiting reduced blood levels of vitamins A, C, and E, β-carotene, and lycopene [123, 124].

Animal Studies. Experimental models of cancer permitted us to understand some of the mechanisms borrowed by vitamins to induce their deleterious effects. The principal and commonly described mechanism was *via* lessening oxidative damage and ROS-induced apoptosis in tumor. In preclinical studies vitamin E, in the form of α-tocopherol, was the most used antioxidant vitamin given its kinetic ability to scavenge certain free radicals ($k \sim 10^5$–$10^6\ M^{-1}\ s^{-1}$) [125]. Vitamin E (100–500 mg/kg) accelerated lung cancer progression in mice through decreasing ROS production and oxidative damage to DNA (i.e., 8-oxoguanine) within tumor [126]. Moreover, vitamin E enhanced the proliferation of lung cancer cells by reducing the expression of the redox-dependent protein p53, which is responsible for cell cycle arrest and apoptosis induction [126]. Vitamins C (8 mg/kg) and E (40 mg/kg) were also able to attenuate the anticancer activity of cisplatin combined with an omega-3 enriched diet, by decreasing lipid peroxidation in lung tumor tissue [127]. Since muscle wasting is a key feature of cancer cachexia, most experimental studies have attempted to use antioxidants with the aim of preventing OS in muscle but did not take into account the redox status of tumor. Although vitamin E was able to attenuate skeletal muscle proteolysis in unloaded mice by reducing muscular OS [128], the use of a mixture containing nutritional doses of vitamins A (0.06 mg/kg), C (11.53 mg/kg), and E (1.73 mg/kg) selectively reduced oxidative damage in C26 tumor and promoted its growth but exacerbated OS within skeletal muscle [17]. Remarkably, these findings may indicate that the use of antioxidant vitamins is more complicated in cachexia-related muscle wasting due to the ambivalence of OS between skeletal muscle and tumor.

6.2. Polyphenols

Clinical Studies. Cachectic patients with head and neck, colon, and lung cancer presented higher ROS levels and low enzymatic antioxidant activity in the blood compared to healthy individuals [129]. Their supplementation with an antioxidant formula containing polyphenols (300 mg/kg) partially reduced systemic OS and improved performance status [15]. Green tea polyphenols (474 mg/day) also attenuated ROS levels in plasma of patients with liver cancer undergoing arterial infusion chemotherapy [130]. A short-term treatment of prostate cancer patients with green tea extracts reduced the circulating levels of prostate-specific antigen (PSA) and vascular endothelial growth factor (VEGF), supporting a potential positive role for polyphenols in cancer prevention and treatment [131].

Animal Studies. Epigallocatechin-3-gallate (EGC-3-G) and theaflavin-3,3′-digallate, found in green and black tea, respectively, were effective in reducing skeletal muscle atrophy caused by cachexia, through inhibiting TNF-α-mediated activation of NF-κB system [132, 133]. Rats bearing Walker 256 tumor receiving daily intraperitoneal (IP) quercetin

injections (10 mg/kg) presented tumor regression and prolonged survival [134]. These beneficial effects of quercetin on tumor growth could be attributed to its antiangiogenic properties, as evidenced by the inhibition of VEGF production in liver extracts [134]. Furthermore, oral quercetin supplementation (25 mg/kg) improved the musculoskeletal function and altered IL-6 production in cachectic $Apc^{Min/+}$ mice, independently of tumor burden [135]. Accordingly, the phosphorylated levels of STAT3 (downstream effector of IL-6) were decreased in skeletal muscle of $Apc^{Min/+}$ mice supplemented with quercetin, while the phosphorylation status of NF-κB remained unchanged [135]. Resveratrol, abundantly found in the skin of grapes, peanuts, and pines, seems to exert its antiwasting effects *in vivo* largely depending on tumor type and the route of administration. Oral resveratrol (200 mg/kg) therapy reduced muscle loss through impairing the DNA binding activity of NF-κB (p65) subunit in both skeletal and cardiac muscles of mice bearing C26 tumor, without influencing tumor growth [136], whereas IP resveratrol injection failed to ameliorate muscle wasting in mice bearing LLC (1 mg/kg) or Yoshida AH-130 (5 and 25 mg/kg) tumor [137]. Although most of these studies found that polyphenols positively affected muscle mass and function, there is a lack of evidence concerning their effects on tumor growth. Globally, tumor weight was the sole parameter used to underscore tumor regression; this data must be consolidated by performing direct analysis on tumor proliferation (e.g., Ki-67, mitotic index), apoptosis, OS, and local inflammation.

6.3. Multimodal Therapy. Since the etiology of cachexia is multifactorial, antioxidants alone cannot fully prevent or reverse muscle atrophy during cachexia. Thus, treatments should be multidimensional to alleviate cachexia symptoms and overcome related sufferance. However, with respect to the topic and aims of the present review, we will discuss in this paragraph only studies that have integrated antioxidants in their treatment arms against cachexia (Table 2). In a randomized phase III study, treatment of gynecological cancer patients with antioxidants, namely, α-lipoic acid and carbocysteine, combined with megestrol acetate (MA, appetite stimulant) and L-carnitine (antioxidant properties) decreased fatigue, circulating TNF-α concentrations, and ROS blood levels, whereas MA alone failed to induce any significant changes in all these parameters [12]. Decidedly, the pioneer work of Mantovani's team clearly indicates that the supplementation of cancer cachectic patients with a cocktail of antioxidants, including polyphenols, vitamins, and cysteine-containing compounds, alone or associated with drugs like MA, L-carnitine, and thalidomide (immunemodulatory function), increased the activity of GPx and reduced ROS levels in blood [15, 138]. Additionally, this combination regimen can effectively ameliorate lean body mass and the performance status in cachectic patients, as assessed by the European cooperative oncology group (ECOG) scale. These clinical positive outcomes could be attributed to the presence of high ROS amounts and the low activity of antioxidant enzymes in blood samples at the baseline. Consistent with this interpretation, the study of Block et al. showed that supplementation with high-doses vitamins C (1000 mg) and E (800 UI) during two months reduced the plasmatic levels of isoprostane (marker of lipid peroxidation), only if it was superior to 50 μg/mL [139]. This indicates the existence of plasmatic critical threshold values for antioxidants and OS biomarkers. When the plasmatic values of antioxidants are inferior to the normal or when blood ROS levels are much higher than healthy control, then antioxidant supplementation will be potentially positive, thence the importance of performing laboratory blood tests in order to determine the antioxidant status before starting intervention. Another interesting detail that may explain the beneficial effects in these trials was, probably, the short duration of treatment going from ten days to four months. Supplementation for a short period seems to be beneficial even when high-doses of antioxidants are used. Furthermore, short-term supplementation was likely to reduce chemotherapy-related toxicity and side-effects in cachectic patients, without affecting its anticancer potential [140].

6.4. Self-Prescription Supplements by Cancer Patients: An Alarming Phenomenon. Patients are highly interested in vitamins and other antioxidant supplements, as they believe that these compounds are natural and beneficial for health [24]. The prevalence of supplements use is approximately 60% in lung, 49% in colon, and 35% in prostate cancer patients [141–143]. Previous studies have shown that the use of alternative medicine was associated with higher education, regular physical activity, fear of cancer recurrence, influence of family members, and participation in social groups [142, 144]. So, *how can this undifferentiated use of supplements by cancer patients impact tumor and anticancer treatment?* As mentioned in previous paragraphs, it seems that supplementation with antioxidants can reduce the efficiency of radiotherapy [24], but the limited number of results from clinical and preclinical studies prevented an evidence-based conclusion. Practitioners usually prohibit the use of supplements during chemotherapy or radiotherapy, as a preventive strategy against an unproven product that could be deleterious for patients' health [145]. Thus, a special attention must be given for cancer patients with comorbidity such as age-related eye disease, since they usually take antioxidant supplements as part of their treatment [24]. To maximize gain, patients not receiving or after achieving radio/chemotherapy, should be monitored for antioxidants use in the context of a well-defined treatment plan [146]. Supplementation with simpler antioxidant mixtures may be also preferred over complex cocktails [146]. Approximately, 50% of patients taking antioxidants or multivitamins did not inform their treating physician; the main reason was that physician did not ask about it [142]. Importantly, patients who discussed the use of supplements were less susceptible to using it [142]. Therefore, clinicians can better control the random use of such compounds by openly discussing with patients about their self-prescription of antioxidants and the potential harms of random use. As illustrated in Figure 4, we suppose that an autoprescription of megadoses antioxidants

Table 2: List of main clinical intervention studies with antioxidants on cachectic patients. One open nonrandomized trial (NRT) shows that, at the baseline, cachectic patients present higher ROS levels and lower GPx activity in blood samples comparing to healthy control subjects. Phases II and III studies show that a combination of antioxidants and other agents, including appetite stimulants (megestrol acetate, MA), anti-inflammatory COX-2 inhibitors (celecoxib, CXB), omega-3 rich fatty acid (eicosapentaenoic acid, EPA), enhancers of lipid β-oxidation (L-carnitine, L-CAR), and immune-modulatory agents (thalidomide, TMD), decreases the levels of ROS in the blood, augment the enzymatic antioxidant activity of GPx, and improve performance status (PS) in cancer cachectic patients. Data are presented in the table as mean values that reached statistical significant difference ($P < 0.05$). No statistically different values are replaced with NSD.

References	Cachectic patient population	Type of study	OS biomarkers (baseline) Cachectic versus healthy control		Treatment		Clinical outcomes (baseline versus treatment)		
			ROS (FORT U)	GPx (U/L)	AO types	Other agents	PS: ECOG score	ROS (FORT U)	GPx (U/L)
Maccio et al. 2012 [12]	Ovary, endometrium, and cervix cancer: 104	R-phase III, 4 mo	—	—	ALA and CS[a]	L-CAR + CXB + MA	1.75 versus 1.12	528 versus 444	6007 versus 7458
Madeddu et al. 2012 [13]	Including H&N, lung, and colorectal: 60	R-phase III, 4 mo	—	—	ALA, CS, PLP, and Vit A, C & E[b]	L-CAR + CXB + MA	1.7 versus 1.4	—	—
Mantovani et al. 2010 [14]	Including breast, pancreas, and colon: 332	R-phase III, 4 mo	—	—	ALA, CS, PLP, and Vit A, C & E[c]	MA + EPA + TMD + L-CAR	2 versus 1.5	NSD	NSD
Mantovani et al. 2006 [15]	Including breast, lung, and stomach: 39	NR-phase II, 4 mo	—	—	ALA, CS, PLP, and Vit A, C & E[c]	EPA + MA + CXB	—	468.5 versus 436.6	NSD
Mantovani et al. 2003 [16]	Including H&N, colon, and lung: 56	NRT, 10 d	403.4 versus 172	6770.6 versus 10813	ALA, CS, NAC, and Vit A, C & E[c]	—	Correlation with OS markers*	403.45 versus 345.9	6770.6 versus 9263.7

[a]α-Lipoic acid (ALA, 600 mg/day) and carbocysteine (CS: 2.7 g/day); [b]N-acetylcysteine (NAC: 1800 mg/day), ALA (200 mg/day), CS (2.7 g/day), Vit A (30000 IU/day), Vit C (500 mg/day), and Vit E (70 mg/day); and [c]polyphenols (PLP: 300–400 mg/day), ALA (300 mg/day), CS (2.7 g/day), Vit A (30000 IU/day), Vit C (500 mg/day), and Vit E (400 mg/day). *Cachectic patients with high ROS levels and low GPx activity exhibited poor performance status. H&N: head and neck cancer.

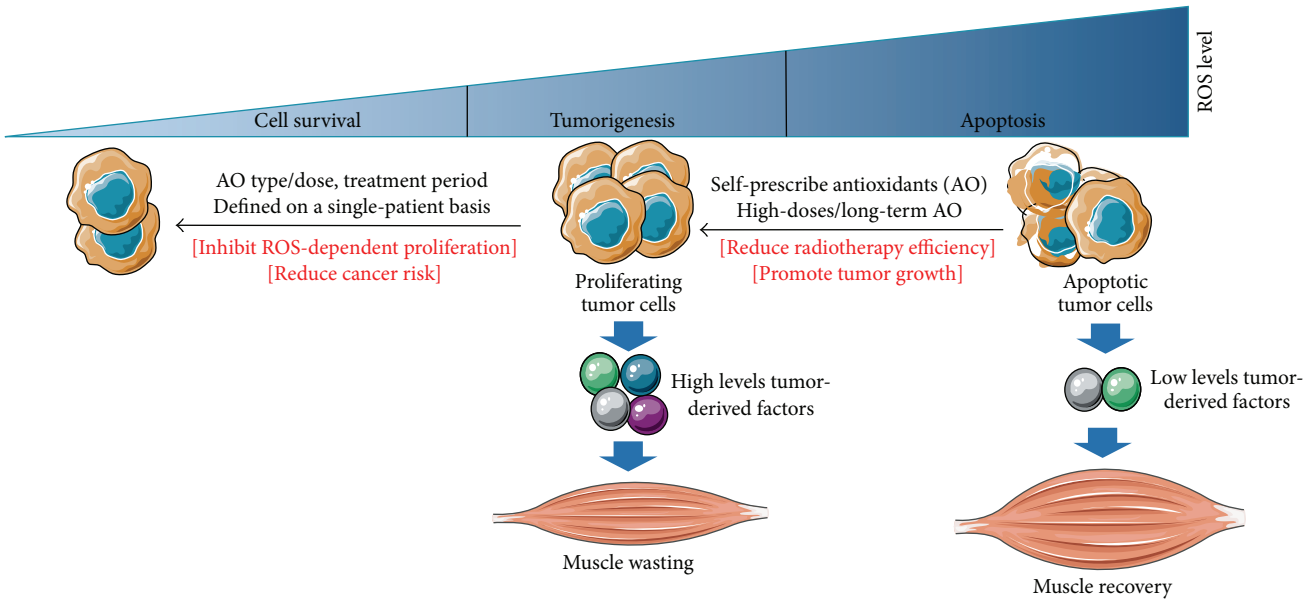

Figure 4: Hypothetical model for the eventual beneficial or deleterious interactions of antioxidants with tumor. ROS play a Janus-faced role by controlling both tumor growth and arrest. The levels of ROS produced within tumor depend on tumor type/localization and whether or not patient is undergoing radio/chemotherapy. Moderate-to-high ROS levels promote tumor proliferation, resulting in an increase in the levels of tumor-derived factors and the subsequent development of muscle atrophy. While high-to-excessive production of ROS activates tumor apoptosis and reduces the related catabolic response, the supplementation with antioxidants may decrease ROS at both systemic and muscular level but could also interact with tumor leading sometimes to undesirable consequences. For example, when excessive levels of ROS are produced within tumor, megadoses of antioxidants, used randomly, could increase tumor proliferation and/or inhibit apoptosis, by reducing oxidative damage in tumor cells. On the other hand, an appropriate use of antioxidants can decrease the risk of cancer development or even slow ROS-dependent cancer growth. The probability of reaping these antioxidant-related benefits could be much higher when supplementation is provided on a single-patient basis.

during a long period could protect tumor and reduce the efficacy of anticancer therapies.

6.5. Exercise: A Good Alternative to Antioxidants in Cancer Patients? Physical activity is well-known to produce moderate levels of ROS and induce hormetic adaptations within skeletal muscle [147]. Adapted activity promotes the expression of antioxidant genes (i.e., *SOD1* and *GPX*) and increases GSH content, which in turn counteract muscular oxidative damage [147]. Additionally, adapted exercise evokes anti-inflammatory responses by producing high amounts of IL-4, IL-10, and IL-15 that antagonize the effects of proinflammatory cytokines and block the activation of the aforementioned procatabolic pathways [148]. We have previously reviewed the impact of physical activity levels on cancer progression and noticed that data available from the literature support a global positive effect of moderate exercise on tumor growth and survival in cancer patients [149]. In 2012, Battaglini and his team proposed their theoretical model of "Exercise Anticachectic Hypothetical (EACH) model." They demonstrated that regular physical activity regimen can positively influence skeletal muscle myoplasticity, in leukemia and breast cancer patients [150, 151]. In other studies, the application of resistance or moderate endurance exercise program improved muscle function and decreased fatigue and proinflammatory cytokines production (i.e., IL-1ra and IL-6) in prostate and breast cancer patients undergoing radiotherapy [152, 153]. Globally, both resistance and endurance exercise improved muscle strength in early stage cancer patients [154]. However, there is a need for clinical trials to determine the effectiveness of exercise in cachectic patients with advanced stages of cancer [155]. It seems that moderate-to-high endurance exercise could be more suitable than resistance exercise to counteract muscle atrophy. In fact, resistance exercise results mainly in the activation of the anabolic Akt/mTOR pathway [156], but the anticachexia role of Akt is still a subject of debate and some experts in the filed consider the activation of Akt useless in the prevention of muscle wasting [157]. Additionally, recent evidence from animal studies suggest that moderate endurance exercise improves muscle mass [158], reduces fatigue, and extends survival, while resistance exercise worsens cachexia symptoms [157, 159]. Endurance is still the most used exercise mode given its capacity to drive metabolic adaptations in skeletal muscle, through activating mitochondrial biogenesis, improving the oxidative capacity of muscle, and increasing antioxidant activity and anti-inflammatory response [147, 160, 161]. Therefore, endurance exercise could be proposed in the early stage of disease for precachectic patients to delay the onset of cachexia and preserve muscle function. It is important to (1) individualize the level of physical activity based on the cardiopulmonary capacity and muscle strength

of patient, (2) if possible, increase the intensity of exercise progressively to reap greater physiological adaptations, and (3) specify the treatment according to the primary end point of the study [162]. For example, a moderate-intensity endurance exercise could be proposed for patients to improve the cardiorespiratory function [163], while high-intensity endurance exercise could be prescribed to induce enzymatic adaptations in skeletal muscle [161]. The capacity of cancer patients with advanced stage of cachexia to perform exercise could be limited owing to anemia and cardiac dysfunction [157]. Thus, exercise could be replaced with other adapted activities such walking in order to avoid further muscle atrophy due to immobilization [164].

7. Conclusive Remarks and Future Directions

Emerging pieces of evidence suggest that the use of antioxidants cannot be standardized for all patients but should be individualized according to patient's need. The administration of high-doses antioxidants for a long period of time was most likely harmful in patients with gastrointestinal, head and neck, and lung cancer, especially if patients were smokers, undergoing radiotherapy, and/or with adequate antioxidant status, while, individuals with antioxidant insufficiency responded positively. In keeping with these findings, the small number of studies performed on cachectic cancer patients exhibiting low antioxidant status or high ROS blood levels indicated that a short-term supplementation (up to six months) was effective in improving physical function and quality of life. Interestingly, it seems that even in the same type of cancer an antioxidant treatment could be more or less advantageous depending on whether the patient is cachectic or not, hence the importance to add cachexia on the list of criteria used to select patients for an antioxidant intervention. In the light of these findings, random complementation cannot prevail. Patients may obtain antioxidants from fruits/vegetables (five portions of 80 g/day), while supplements must be reserved for those with particular needs. Accordingly, the measurement of blood antioxidant levels could be a simple approach to identify patients with specific deficiencies and, therefore, improve the use of such compounds in cancer cachexia. We might underscore that, given the multiorgan presence of OS in cancer cachexia, systemic antioxidant status does not necessarily reflect the redox events occurring in other organs like muscle, and the absence of antioxidant deficiency or high ROS rates in blood does not mean that muscles are spared from oxidative damage and atrophy. Nonetheless, this method remains more appropriate to the clinical context nowadays, where performing skeletal muscle biopsies is restricted for ethical and methodological reasons. Based on literature, natural polyphenols appear to be more effective than vitamins in cancer cachexia, probably, due to their capacity to modulate redox status, epigenetic pathways, and cellular senescence [165]. Furthermore, adapted physical activity could be a promising strategy for cachectic patients, as it positively affects muscle performance, OS parameters, and systemic inflammation. However, there is a real need for new clinical studies on a larger scale to further explore the role of antioxidants and physical activity in cancer cachexia. At the moment, a regimen combining moderate physical activity with an appropriate nutritional care could be the optimal way to improve quality of life, preserve muscle endurance, and naturally ameliorate enzymatic antioxidant defense in cancer cachectic patients.

Competing Interests

The authors of this paper declare no conflict of interests.

Funding

Mohamad Assi is a recipient of a Ph.D. research fellowship (no. 2012/22) from the Brittany Region Council. This work was supported by the University of Rennes 2.

Acknowledgments

The authors thank Dr. Frédéric Derbré and Dr. Nicolas Pierre for helpful discussions.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5293284, 10 pages
http://dx.doi.org/10.1155/2016/5293284

Review Article

Roles of Reactive Oxygen Species in Anticancer Therapy with *Salvia miltiorrhiza Bunge*

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Received 6 March 2016; Revised 16 June 2016; Accepted 27 June 2016

Academic Editor: Pavel P. Philippov

Cancer is a leading cause of death worldwide. We aim to provide a systematic review about the roles of reactive oxygen species (ROS) in anticancer therapy with *Salvia miltiorrhiza* Bunge (Danshen). Danshen, including its lipophilic and hydrophilic constituents, is potentially beneficial for treating various cancers. The mechanisms of ROS-related anticancer effects of Danshen vary depending on the specific type of cancer cells involved. Danshen may enhance TNF-α-induced apoptosis, upregulate caspase-3, caspase-8, caspase-9, endoplasmic reticulum stress, P21, P53, Bax/Bcl-2, DR5, and AMP-activated protein kinase, or activate the p38/JNK, mitogen-activated protein kinase, and FasL signaling pathways. Conversely, Danshen may downregulate human telomerase reverse transcriptase mRNA, telomerase, survivin, vascular endothelial growth factor/vascular endothelial growth factor receptor 2, CD31, NF-κB, Erk1/2, matrix metalloproteinases, microtubule assembly, and receptor tyrosine kinases including epidermal growth factor receptors, HER2, and P-glycoprotein and inhibit the PI3K/Akt/mTOR or estrogen receptor signaling pathways. Therefore, Danshen may inhibit cancer cells proliferation through antioxidation on tumor initiation and induce apoptosis or autophagy through ROS generation on tumor progression, tumor promotion, and tumor metastasis. Based on the available evidence regarding its anticancer properties, this review provides new insights for further anticancer research or clinical trials with Danshen.

1. Introduction

Cancer is a leading cause of mortality throughout the world. In addition to conventional therapies such as surgery, chemotherapy, and radiotherapy, traditional Chinese medicine and other complementary or alternative therapies may be necessary for cancer patients [1]. *Salvia miltiorrhiza Bunge* (Danshen) has been used widely for the treatment of various diseases [2–7] including cancers [8–12] for thousands of years within the China community. Danshen, a Chinese herbal medicine, contains two major groups of chemicals [12–15]. The first group includes lipophilic compounds such as tanshinone I, tanshinone IIA, acetyltanshinone IIA, cryptotanshinone, isocryptotanshinone, dihydrotanshinone,

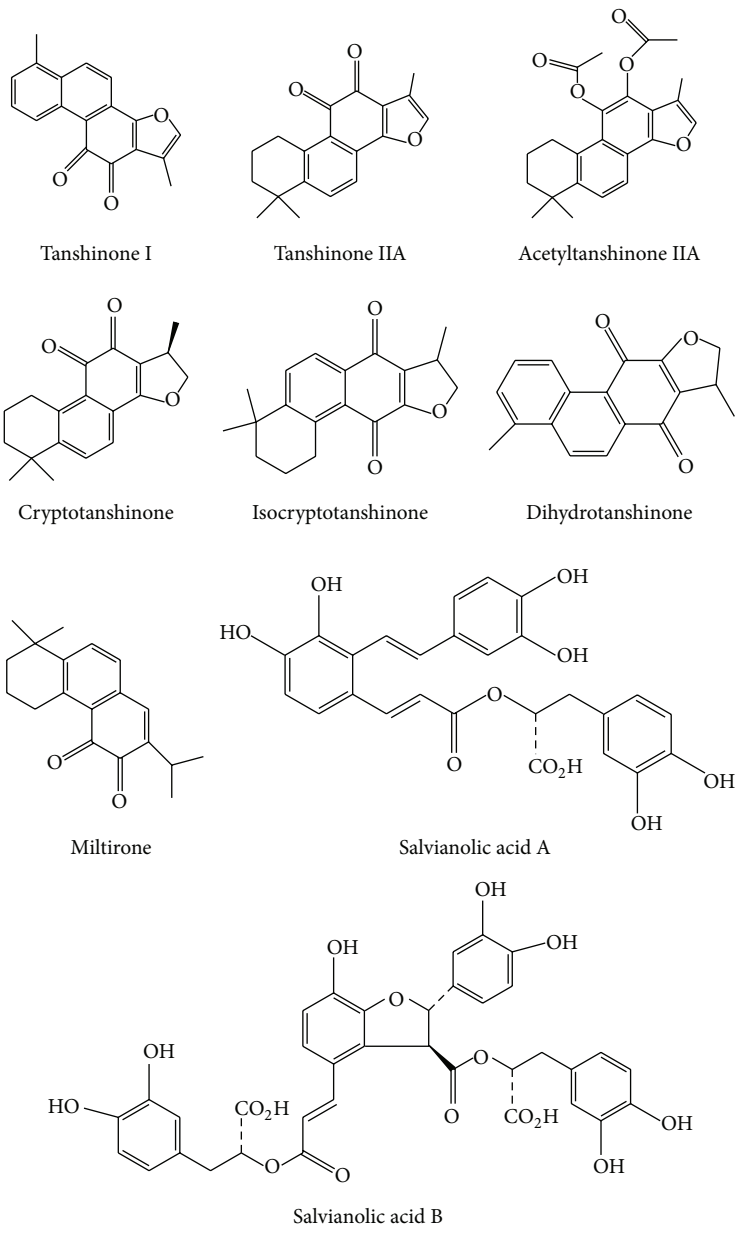

Figure 1: Chemical structures of the different constituents of Danshen. Danshen contains lipophilic compounds including tanshinone I, tanshinone IIA, acetyltanshinone IIA, cryptotanshinone, isocryptotanshinone, dihydrotanshinone, and miltirone. Danshen also contains hydrophilic phenolic acids including salvianolic acids A and B.

15,16-dihydrotanshinone I, and miltirone. The second group includes the hydrophilic phenolic acids such as salvianolic acids A and B (Figure 1). Our research and numerous other publications have demonstrated that both groups of Danshen compounds may have anticancer effects (Tables 1 and 2). This systematic review provides an appraisal of the roles of reactive oxygen species (ROS) in cancer biology and anticancer therapy with Danshen. Based on the evidence demonstrating anticancer properties of Danshen and the roles of ROS in cancer biology, this review summarizes current data regarding the ROS-related anticancer effects of Danshen components and brings new insights for further anticancer research or clinical trials with this traditional Chinese herb.

2. Methods

Keyword searches were done using the combined terms "reactive oxygen species and cancer and Danshen" or "reactive oxygen species and cancer and *Salvia miltiorrhiza*". These searches were done using the Medicine, PubMed, EMBASE, Cochrane library, CINAHL, and Scopus databases. The contents of the identified articles were summarized and the current review focused on the ROS-related anticancer

Table 1: Lipophilic components from *Salvia miltiorrhiza* that modify ROS-related effects on cancer cells.

Components [reference]	Cancer cells	Effects
Tanshinones [16]	Lung cancer 95D cells	Induces apoptosis and prosurvival autophagy mediated by increasing the formation of intracellular ROS
Tanshinone I [17]	Prostate cancer cells	Enhances TRAIL via upregulation of miR-135a-3p-mediated death receptor 5
Tanshinone I [18]	Human breast cancer MDA-MB-453 cells	Induces antiproliferative activity and cell cycle arrest by inhibiting the PI3K/Akt/mTOR signaling pathways
Tanshinone I [19]	Leukemia U937 THP-1 and SHI 1 cells	Induces apoptosis by activating caspase-3 and decreasing hTERT mRNA expression and telomerase activity, as well as downregulating survivin expression
Tanshinone IIA [20]	Prostate cancer cells	Induces apoptosis and autophagy that depends on intracellular ROS production
Tanshinone IIA [21]	Gastric cancer cells	Suppresses cell growth by blocking glucose metabolism
Tanshinone IIA [22]	Human non-small cell lung cancer A549 cells	Decreases VEGF/VEGFR2 expression and induces apoptosis and cell cycle arrest at the S phase
Tanshinone IIA [23]	Human oral cancer KB cells	Induces apoptosis through the mitochondria-dependent pathway in which there is a loss of the mitochondrial membrane potential and activation of caspase-3 and caspase-9
Tanshinone IIA [24]	Human colon cancer cells	UDP-glucuronosyltransferase 1A compromises the intracellular accumulation and resultant apoptotic effect of tanshinone IIA
Tanshinone IIA [25]	Cervical cancer CaSki cells	Inhibits cell growth by activating ER stress pathways and promoting caspase cascades with concomitant upregulation of p38 and JNK phosphorylation and signaling
Tanshinone IIA [26]	Human hepatoma J5 cells	Increases Bax and caspase-3 and decreases CD31 expression
Tanshinone IIA [27]	Non-small cell lung cancer H596 cells	Activates ROS-triggered, p53-independent, and caspase-dependent mitochondrial apoptotic cell death pathway
Tanshinone IIA [28]	786-O human renal cell carcinoma cells	Induces apoptosis by activating p53 expression and subsequently upregulating p21 and Bax
Tanshinone IIA [29]	Leukemia U937 cells	Induces apoptosis by activating PXR, which suppresses the activity of NF-κB
Tanshinone IIA [30]	human non-small lung cancer A549 cells	Induces apoptosis by increasing ROS and the ratio of Bax/Bcl-2 and then decreasing the mitochondrial membrane potential, which leads to cytochrome c release
Tanshinone IIA [31]	Small cell lung cancer H146 cells	Inhibits cell growth by upregulating the Bax/Bcl-2 ratio and decreasing the mitochondrial membrane potential
Tanshinone IIA [32]	Cervical cancer HeLa cells	Inhibits cell growth by interfering with the process of microtubule assembly, leading to G2/M phase arrest and subsequent apoptosis
Acetyltanshinone IIA [33]	Breast cancer	Induces G1/S phase arrest and apoptosis by downregulating the receptor tyrosine kinases EGFR/HER2 and activating AMP-activated protein kinase
Acetyltanshinone IIA [34]	Breast cancer cells	Induces ROS generation and Bax translocation to mitochondria, resulting in mitochondrial damage, cytochrome c release, caspase-3 activation, and apoptotic cell death
Cryptotanshinone [35]	Breast cancer cells	Suppresses estrogen receptor signaling

Table 1: Continued.

Components [reference]	Cancer cells	Effects
Cryptotanshinone [36]	Acute lymphoblastic leukemia cells	Inhibits cellular movement and induces G2/M cell cycle arrest and apoptosis
Cryptotanshinone [37]	Lung cancer cells	Induces prodeath autophagy through JNK signaling that is mediated by ROS generation
Cryptotanshinone [38]	HepG2 hepatoma	Induces G1 cell cycle arrest and autophagic cell death by activating the AMP-activated protein kinase signaling pathway
Cryptotanshinone [39]	A375 melanoma cells	Restores sensitivity in cancer cells that are resistant to TRAIL by upregulating DR5 expression
Cryptotanshinone [40]	Rh30 human rhabdomyosarcoma; DU145 prostate carcinoma; and human MCF-7 breast cancer cells	Induces ROS, thereby activating p38/JNK and inhibiting Erk1/2 leading to caspase-independent cell death
Cryptotanshinone [41]	Neuro-2a cells	Inhibits sodium nitroprusside-induced apoptosis by antioxidant effects and regulating the NF-κB and MAPK pathways
Cryptotanshinone [42]	HepG2 hepatoma and MCF-7 breast cancer cells	Induces ER stress-mediated apoptosis
Cryptotanshinone [43]	Prostate cancer cells	Suppresses androgen receptor- (AR-) mediated growth by blocking AR dimerization and formation of the AR-coregulator complex
Cryptotanshinone [44]	Chronic myeloid leukemia KBM-5 cells	Enhances TNF-α-induced apoptosis through ROS-dependent activation of caspase-8 and p38
Isocryptotanshinone [45]	Human breast cancer MCF-7 cells	Induces apoptosis and activates MAPK signaling pathways
Dihydrotanshinone [46]	HepG2 cells	Activates ROS-mediated phosphorylation of p38 MAPK
Dihydrotanshinone I [47]	Colon cancer	Induces caspase- and ROS-dependent apoptosis and autophagy
15,16-Dihydrotanshinone I [48]	Human HL-60 Leukemia Cells	Induces apoptosis through activation of the JNK and FasL signaling pathways
Miltirone [49]	Human hepatoma HepG2 cells	Activates caspase-dependent apoptotic pathways and triggers ROS-mediated MAPK signaling pathways
Miltirone [50]	Acute lymphoblastic leukemia cells	Induces G2/M cell cycle arrest and apoptosis

Table 2: Hydrophilic components from *Salvia miltiorrhiza* that modify ROS-related effects on cancer cells.

Components [reference]	Cancer cells	Effects
Salvianolic acid A [51]	MCF-7 breast cancer cells	Downregulates the level of P-glycoprotein and triggers apoptosis, which is associated with increased caspase-3 activity, disrupted mitochondrial membrane potential, downregulated Bcl-2 expression, and upregulated Bax expression in resistant cells
Salvianolic acid A [52]	Human neuroblastoma SH-SY5Y cells	Prevents 1-methyl-4-phenylpyridinium ion-induced cytotoxicity, which may be ascribed to its antioxidant properties and antiapoptotic activity via regulating the expression of Bcl-2 and Bax
Salvianolic acid B [53]	Human glioma U87 cells	Induces apoptosis through p38-mediated ROS generation
Salvianolic acid B [54]	Human neuroblastoma SH-SY5Y cells	Prevents 1-methyl-4-phenylpyridinium-induced apoptosis by relieving oxidative stress and modulating the apoptotic process
Salvianolic acid B [55]	Human neuroblastoma SH-SY5Y cells	Prevents dopamine-induced apoptosis that may be mediated by the ROS and the Erk and Bcl-2 pathways

effects of Danshen. After removing duplicate publications and excluding information that was unrelated to ROS, we collected 39 articles about the ROS-related anticancer effects of Danshen. These publications included a consideration of 34 lipophilic and 5 hydrophilic compounds isolated from Danshen.

3. Role of ROS in Cancer

Carcinogenesis is a progressive process from normal to cancerous cells. Reactive oxygen species (ROS) are closely related to carcinogenesis and play an important role in cancer. Previous studies have shown that ROS may be involved in multistep tumorigenesis including tumor initiation and transformation, tumor progression, tumor promotion, tumor angiogenesis, and tumor metastasis [56–58]. ROS are generated by both mitochondria and NADPH oxidases. Oxidative stress results from the generation of free radicals such as the superoxide anion, perhydroxyl radical, hydroxyl radical, and nitric oxide, as well as other nonradical but reactive species such as hydrogen peroxide, singlet oxygen, hypochlorous acid, and peroxynitrite [56, 57].

Mitochondria in malignant cells are characterized by the overproduction of ROS and differ structurally and functionally from those in normal cells [59]. A major source of ROS is oxidative metabolism in the mitochondria of eukaryotic cells. In normal cells, low-level concentrations of ROS, related to mitochondrial electron transport activity, are required for many cellular processes and signal transduction. Cancer cells generate more ROS as compared to normal cells. The increased generation of ROS in cancer cells may alter mitochondrial metabolism [59, 60] and disturb cellular signaling pathways [61, 62] that are mediated through the transcription factors NF-κB and STAT3, hypoxia-inducible factor-1α, kinases, growth factors, cytokines, and other enzymes [63].

ROS can induce cellular DNA damage and DNA methylation [64] resulting in mutations, which causes healthy cells to transform into malignant cells. Some cancer cells overexpress the ROS-producing NADPH oxidases and ROS-removing antioxidant enzymes. Conversely, there is also evidence showing that excess ROS can result in cancer cell death through autophagy [65, 66] and/or apoptosis [62, 67]. Cancer cells may be more sensitive than normal cells to the overproduction of ROS. Thus, increasing oxidative stress by generating ROS exogenously may be selective for cancer cells without affecting normal cells [68, 69].

4. Lipophilic Components of Danshen

Tanshinone I, tanshinone IIA, acetyltanshinone IIA, cryptotanshinone, isocryptotanshinone, dihydrotanshinone, and miltirone are the main lipid-soluble potential anticancer constituents of Danshen. These compounds have shown anticancer activity (Table 1) with remarkable dose- and time-dependent inhibitory effects on the viability on prostate, lung, breast, leukemia, gastric, oral, colon, cervical, hepatoma, renal, melanoma, rhabdomyosarcoma, and neuroblastoma cancer cells. These effects, in terms of ROS, are described in more detail for each cell type in the following sections.

4.1. ROS-Related Anticancer Effects of Tanshinones on Prostate Cancer Cells. Tanshinone I enhanced tumor necrosis factor-(TNF-) related apoptosis inducing ligand (TRAIL) via increasing cleaved poly-ADP ribose polymerase (PARP), arresting cells in the subG1 phase, activating caspase-8 and caspase-9, and upregulating miR-135a-mediated death receptor 5 [17]. The induction of apoptosis and autophagy by tanshinone IIA was dependent on intracellular ROS production [20]. Cryptotanshinone suppressed androgen receptor-mediated cell growth [43] and induced ROS thereby phosphorylating (i.e., activating) P38/JNK and inhibiting Erk1/2, resulting in caspase-independent death in DU145 prostate cancer cells [40].

4.2. ROS-Related Anticancer Effects of Tanshinones on Lung Cancer Cells. Tanshinones inhibited the proliferation of 95D lung cancer cells by increasing caspase-3 activity and inducing apoptosis and prosurvival autophagy [16], through the increased generation of intracellular ROS. Tanshinone IIA decreased vascular endothelial growth factor/vascular

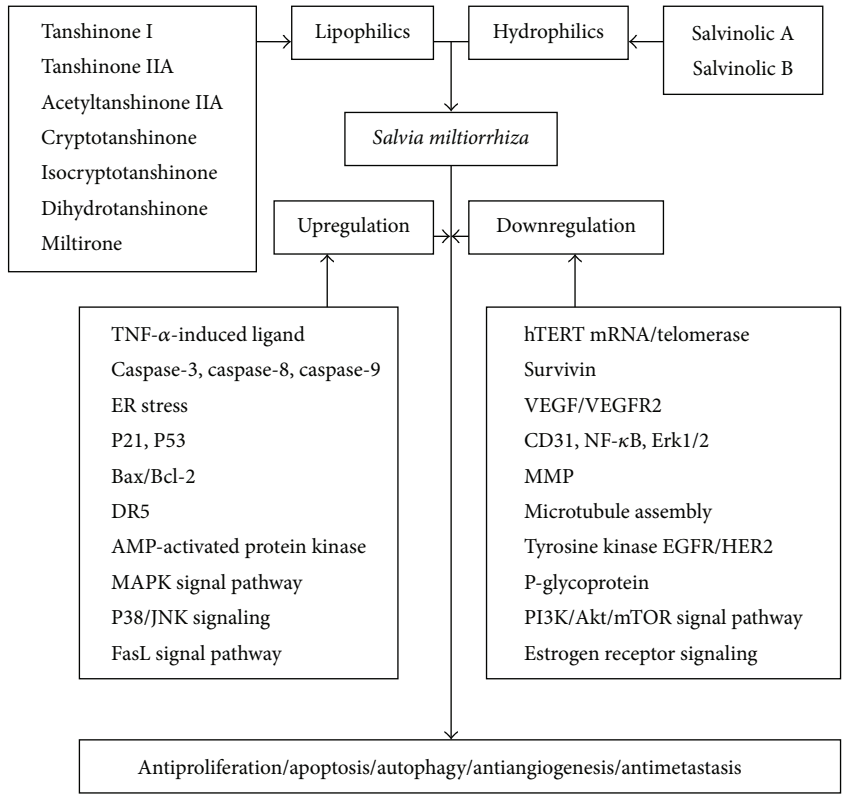

Figure 2: Schematic diagram of ROS-related anticancer effects mediated by Danshen. Upregulation: TNF-α, caspase-3, caspase-8, caspase-9, endoplasmic reticulum (ER) stress, P21, P53, Bax/Bcl-2, DR5, AMP-activated protein kinase, MAPK signaling pathways, the phosphorylation (activation) of p38/JNK signaling, and the FasL signaling pathway. Downregulation: hTERT mRNA, telomerase, survivin, VEGF/VEGFR2, CD31, NF-κB, Erk1/2, MMP, microtubule assembly, tyrosine kinases such as EGFR/HER2, P-glycoprotein, and PI3K/Akt/mTOR, and estrogen receptor signaling.

endothelial growth factor receptor 2 (VEGF/VEGFR2) expression and induced apoptosis with cell cycle arrest at the S phase in human non-small cell lung cancer A549 cells [22]. These researchers noted that tanshinone IIA activated a ROS-induced, P53-independent [27], and caspase-dependent mitochondrial apoptotic cell death pathway that was characterized by an increased ratio of Bax to Bclx1 or Bax to Bcl-2, decreased mitochondrial membrane potential [30], caspase activation, PARP-1 cleavage, and cytochrome c release in A549 cells and small cell lung cancer H146 cells [31]. Cryptotanshinone also induced ROS-mediated prodeath autophagy through JNK signaling [37].

4.3. ROS-Related Anticancer Effects of Tanshinones on Breast Cancer Cells. Tanshinone I downregulated the PI3K/Akt/mTOR signaling pathway, induced cell cycle arrest, and inhibited the proliferation of breast cancer MCF-7 and MDA-MB-453 cells [18]. Acetyltanshinone IIA induced G1/S phase arrest and apoptosis with downregulation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR)/HER2 and activated AMP-activated protein kinase (AMPK) [33]. Acetyltanshinone IIA also induced ROS generation and Bax translocation to mitochondria resulting in mitochondrial damage, cytochrome c release, caspase-3 activation, and apoptotic cell death in HER2 positive breast cancer cells [34]. Cryptotanshinone suppressed estrogen receptor signaling and induced endoplasmic reticulum (ER) stress-mediated apoptosis [42] and ROS generation, activating P38/JNK and inhibiting Erk1/2. This led to caspase-independent cell death in MCF-7 breast cancer cells [40]. Isocryptotanshinone induced apoptosis and activated the mitogen-activated protein kinase (MAPK) signaling pathway in MCF-7 breast cancer cells [45].

4.4. ROS-Related Anticancer Effects of Tanshinones on Leukemia Cells. Some researchers found that tanshinone I activated caspase-3 and decreased human telomerase reverse transcriptase (hTERT) mRNA expression and telomerase activity, as well as downregulating survivin expression, in monocytic leukemia U937 THP-1 and SHI 1 cells [19]. Another study reported that tanshinone IIA induced apoptosis through the activation of PXR, which suppressed NF-κB activity in leukemia U937 cells [29].

Cryptotanshinone inhibited cellular movement and induced G2/M phase arrest in acute lymphoblastic leukemia cells [36]. Another study revealed that cryptotanshinone enhanced TNF-α-induced apoptosis through the ROS-dependent activation of caspase-8 and p38 in chronic myeloid leukemia KBM-5 cells [44]. 15,16-Dihydrotanshinone I induced apoptosis through activation of the JNK and FasL

signaling pathways in human HL-60 leukemia cells [48]. Miltirone induced G2/M cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells [50].

4.5. ROS-Related Anticancer Effects of Tanshinones on Oral, Gastric, and Colon Cancer Cells. One previous study reported that tanshinone IIA induced apoptosis through the mitochondria-dependent pathway with the loss of mitochondrial membrane potential and the activation of caspase-9 and caspase-3 in human oral cancer KB cells [23]. Another study that examined the effects of tanshinone IIA reported that it suppressed cell growth by blocking glucose metabolism in gastric cancer cells [21]. Another article revealed that UDP- glucuronosyltransferase 1A compromised the apoptotic effects of tanshinone IIA by reducing its intracellular exposure and switching the NAD(P)H: quinine oxidoreductase 1-triggered redox cycle to metabolic elimination [24]. Other research noted that dihydrotanshinone I induced caspase and ROS-dependent apoptosis and autophagy in colon cancer cells [47].

4.6. ROS-Related Anticancer Effects of Tanshinones on Cervical Cancer Cells. Our previous studies showed that tanshinone IIA had anticancer effects on typical cervical HeLa and advanced cervical CaSki cancer cells. Tanshinone IIA induced apoptosis by interfering with the microtubule assembly process, leading to G2/M phase arrest and subsequent apoptosis in HeLa cells [32]. It also appeared to inhibit cell growth through activating the ER stress pathway and promoting caspase cascades with concomitant upregulation of the phosphorylation of the p38 and JNK-Bax-caspase-3/9 signaling pathways (Figure 3) in CaSki cells [25].

4.7. ROS-Related Anticancer Effects of Tanshinones on Hepatoma Cells. Tanshinone IIA increased Bax and caspase-3 levels and decreased CD31 expression in human hepatoma J5 cells [26]. Cryptotanshinone induced ER stress-mediated apoptosis [42] and induced G1 cell cycle arrest and autophagic cell death by activating the AMPK signaling pathway [38]. Dihydrotanshinone activated ROS-mediated phosphorylation of p38 MAPK in HepG2 cells [46]. Miltirone activated the caspase-dependent apoptotic pathway and triggered the ROS-mediated MAPK signaling pathway in human hepatoma HepG2 cells [49].

4.8. ROS-Related Anticancer Effects of Tanshinones on Renal Carcinoma Cells, Melanoma, Neuroblastoma, and Rhabdomyosarcoma Cells. Previous research noted that tanshinone IIA induced apoptosis in renal carcinoma cells by activating p53 expression and subsequently inducing the upregulation of p21 and Bax [28].

The other cryptotanshinone would restore the sensitivity of A375 melanoma cells that were resistant to TRAIL by upregulating the expression of death receptor 5 (DR5) [39]. It also could inhibit sodium nitroprusside-induced apoptosis by an antioxidant effect and by regulating NF-κB and the MAPK pathway in Neuro-2a cells [41]. Cryptotanshinone was reported to induce ROS, then activate P38/JNK, and inhibit Erk1/2 in rhabdomyosarcoma cells. These effects then led to caspase-independent cell death in these cells [40].

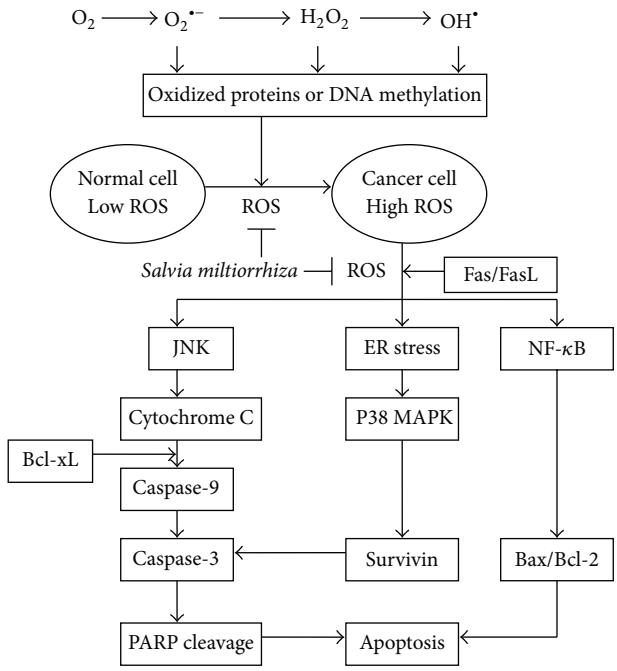

Figure 3: Schematic diagram of effects of *Salvia miltiorrhiza* on reactive oxygen species-related apoptosis of cancer cells.

5. ROS-Related Anticancer Effects of Hydrophilic Components Found in Danshen

Polyphenols, as dietary antioxidants, are most abundant in fruits, vegetables, and cereals [70, 71]. Numerous clinical studies, as well as *in vitro* and *in vivo* experiments, have strongly supported the ability of polyphenols to reduce the risk of many cancers. Some antioxidant polyphenols can downregulate TNF and might be useful as mitochondrially targeted anticancer drugs [72–74].

Salvianolic acids A and B are the main water-soluble polyphenolic derivatives found in Danshen. Similar to other natural polyphenols, they have potential anticancer effects (Table 2). Salvianolic acid A elevated ROS levels, downregulated P-glycoprotein, and triggered apoptosis by increasing caspase-3 activity and upregulating Bax expression, while downregulating Bcl-2 expression and disrupting the mitochondrial membrane potential in multidrug resistance MCF-7 human breast cancer cells [51]. Other research showed that salvianolic acids A and B had antioxidant and antiapoptotic properties that were involved in protecting SH-SY5Y human neuroblastoma cells against 1-methyl-4-phenylpyridinium ion-induced mitochondrial dysfunction. This dysfunction was characterized by loss of the mitochondrial membrane potential, condensation of nuclei, cytochrome c release, and increases in the Bax/Bcl-2 ratio [52, 54]. Salvianolic acid B prevented 6-hydroxydopamine-induced apoptosis in SH-SY5Y cells by reducing the increase of caspase-3 activity and the translocation of cytochrome c into the cytosol from

mitochondria [55]. Another study revealed that salvianolic acid B induced apoptotic cell death in human glioma U87 cells through p53 and the phosphorylation and activation of p38 MAPK to increase ROS generation [53].

6. Conclusion

Danshen may be a potential complementary or alternative therapy for various cancer patients. We found the potential utility of this natural product, or its active constituents including lipophilic compounds such as tanshinone I, tanshinone IIA, acetyltanshinone IIA, cryptotanshinone, isocryptotanshinone, dihydrotanshinone, 15,16-dihydrotanshinone I, miltirone, and hydrophilic phenolic acids such as salvianolic acids A and B (Figure 1). The ROS-related anticancer effects of the lipophilic and hydrophilic constituents isolated from Danshen vary, depending on the specific type of cancer cells (Tables 1 and 2). Overall, Danshen can suppress cell proliferation through antioxidation on tumor initiation and induce apoptosis (Figure 3) or autophagy through ROS generation on tumor progression, tumor promotion, and tumor metastasis. Some components of Danshen may enhance TNF-α-induced apoptosis and upregulate caspase-3, caspase-8, caspase-9, ER stress, P21, P53, Bax/Bcl-2, DR5, and AMPK and activate the p38/JNK, MAPK, or FasL signaling pathways. Conversely, these compounds can downregulate hTERT mRNA, telomerase, survivin, VEGF/VEGFR2, CD31, NF-κB, Erk1/2, MMPs, microtubule assembly, tyrosine kinases such as EGFR/HER2 and P-glycoprotein and inhibit the PI3K/Akt/mTOR or estrogen receptor signaling pathways (Figure 2). Combined, these effects inhibit cancer cell proliferation by arresting cell cycle progression, inducing cancer cell apoptosis and/or autophagy, and exerting antiangiogenic and antimetastatic effects. However, in accordance with laboratory evidences obtained *in vitro* and *in vivo*, rigorous human studies are needed to demonstrate the anticancer effects of Danshen. Future well-designed clinical studies, such as randomized controlled clinical trials, will be necessary to confirm the efficacy of Danshen as an anticancer agent in human patients.

Competing Interests

Authors declare no competing interests.

Acknowledgments

This work was supported by a grant from the National Science Council [NSC102-2628-B-182-003-MY3] and Chang Gung Memorial Hospital [CMRPD1B0401~2, CMRPD1D0341~2, and BMRP445], Taiwan

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1580967, 10 pages
http://dx.doi.org/10.1155/2016/1580967

Review Article

Reactive Oxygen Species Regulate T Cell Immune Response in the Tumor Microenvironment

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Received 18 March 2016; Revised 6 June 2016; Accepted 30 June 2016

Academic Editor: Svetlana Karakhanova

Reactive oxygen species (ROS) produced by cellular metabolism play an important role as signaling messengers in immune system. ROS elevated in the tumor microenvironment are associated with tumor-induced immunosuppression. T cell-based therapy has been recently approved to be effective for cancer treatment. However, T cells often become dysfunctional after reaching the tumor site. It has been reported that ROS participate extensively in T cells activation, apoptosis, and hyporesponsiveness. The sensitivity of T cells to ROS varies among different subsets. ROS can be regulated by cytokines, amino acid metabolism, and enzymatic activity. Immunosuppressive cells accumulate in the tumor microenvironment and induce apoptosis and functional suppression of T cells by producing ROS. Thus, modulating the level of ROS may be important to prolong survival of T cells and enhance their antitumor function. Combining T cell-based therapy with antioxidant treatment such as administration of ROS scavenger should be considered as a promising strategy in cancer treatment, aiming to improve antitumor T cells immunity.

1. Introduction

Reactive oxygen species (ROS) are small short-live oxygen-containing molecules that are chemically highly reactive. Of more than 20 types of ROS, superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals are the most important subtypes contributing to cell damage and even death [1]. ROS are generated mainly by following mechanisms: xanthine oxidase-dependent, respiratory chain and NADPH oxidase- (NOX-) dependent pathways. Mitochondrium is a major place to produce intracellular ROS, and complexes I and III of electron transport systems are main sources of mitochondrial $O_2^{\bullet-}$ [2]. In addition, there are also other exogenous sources of ROS, including ultraviolet and gamma radiation, air pollutants, and chemicals [3–5]. Superoxide anion generated initially in cell is converted rapidly into H_2O_2 freely crossing cell membranes, which can be further converted into hydroxyl radicals in the presence of Fe^{2+} or Cu^{2+} [6]. Compared to other ROS, H_2O_2 has a longer half-life (about 1 ms in an aqueous solution) than other ROS (<1 μs) and functions as an important oxidant in microenvironment [7, 8]. Moreover, H_2O_2 reacts with thiols at a physiological concentration and forms disulfide bond [9]. Thus, H_2O_2 can act as a second messenger because of the following: (i) it has relative long half-life, (ii) it is uncharged, (iii) it can cross membranes, (iv) it is relatively specific (thiols), and the modifications (disulfide bonds) are reversible [10]. H_2O_2 has been reported to participate in many processes, such as cell growth, stem cell renewal, tumorigenesis, cell death, cell senescence, cell migration, oxygen sensing, angiogenesis, circadian rhythm maintenance, myofibroblasts differentiation, and immune responses [7, 11–16].

ROS elevated in almost all cancers act as a double-edged sword during tumor development [17]. For example, ROS-mediated DNA damage triggers malignant transformation of cells and promotes cancer initiation. ROS levels are also

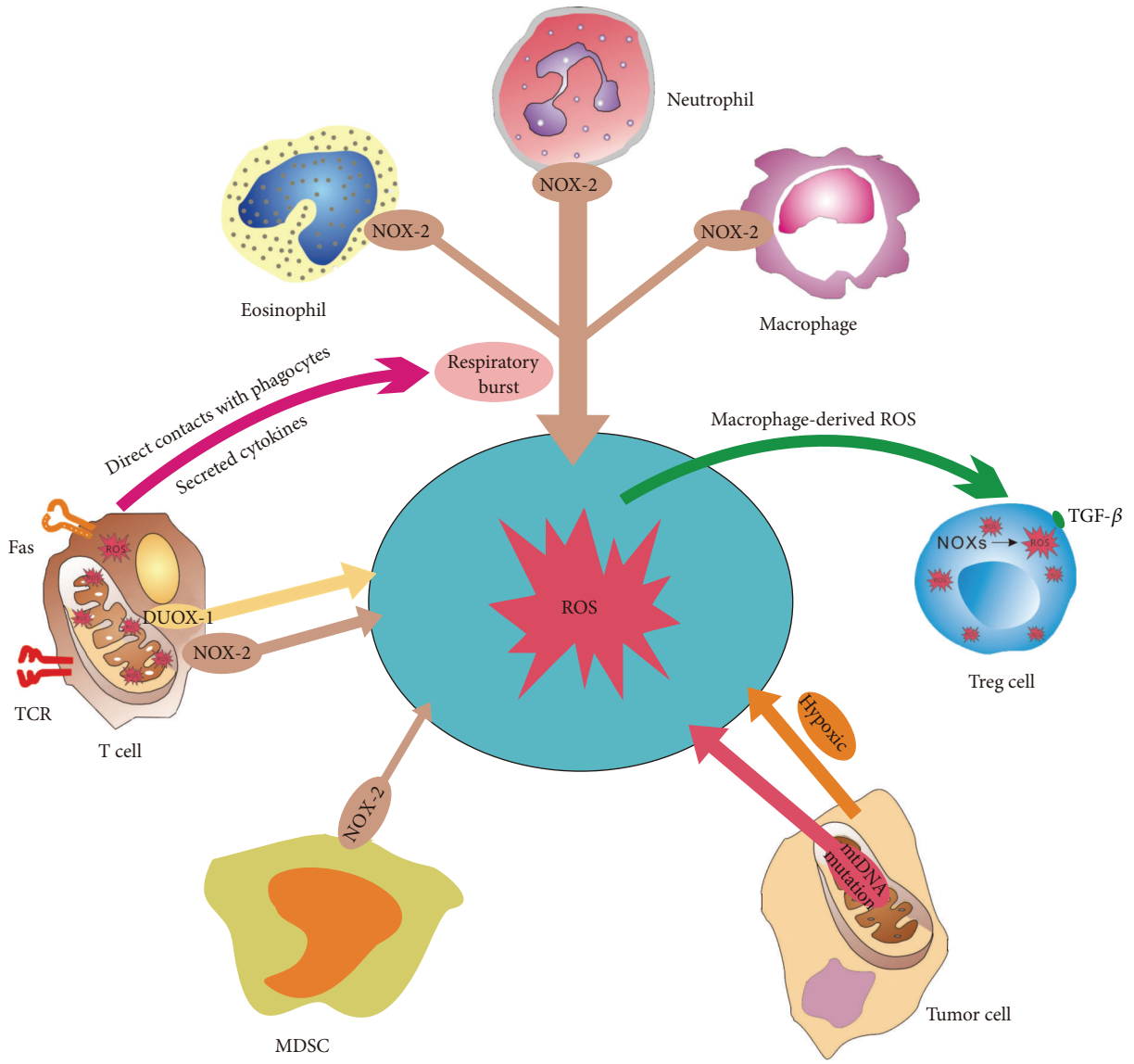

Figure 1: ROS produced in the tumor microenvironment. FasL ligation and TCR signaling in T cells could induce the production of ROS via NOX-2, DUOX-1, and mitochondria. Activated phagocytes (neutrophils, eosinophils, and mononuclear phagocytes) can produce large amounts of ROS by the NOX-2 during respiratory burst. Activated T cells can also induce respiratory burst by direct contacts with phagocytes or cytokines. TGF-β activates NOXs of Tregs, which trigger the production of ROS. Moreover, macrophage-derived ROS can induce Tregs accumulation in the tumor microenvironment. Mutations of mitochondrial DNA (mtDNA) in tumor cells result in a deficiency in respiratory complex I activity and contribute to the overproduction of ROS. MDSCs also produce amounts of ROS in the tumor microenvironment.

associated with cancer cell stemness [18]. It has been demonstrated that immunosuppressive tumor microenvironment facilitates tumor invasion, metastasis, and resistance [19]. ROS are likely immunosuppressive participants in tumor progression [20]. Indeed, ROS production greatly contributes to inhibitory activities of tumor-induced-immunosuppressive cells [21, 22]. Therefore, ROS are not only mediators of oxidative stress, but also players of immune regulation during tumor development. ROS-mediated signaling can be additionally regulated via altering local concentrations (e.g., using antioxidants) [23]. ROS are essential particularly at low levels for a wide range of innate immune functions, including antiviral, antibacterial, and antitumor responses [24]. This review will mainly discuss the production of ROS in the tumor microenvironment and the impact on antitumor T cell immune response.

2. ROS Generation in the Tumor Microenvironment

As shown in Figure 1, ROS produced by cancer cells and tumor-infiltrating leukocytes, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs), can suppress the immune responses.

It has been revealed that MDSCs, as one of the major immunosuppressive subsets, play a pivotal role in promoting tumor progression and contribute to suppressive tumor microenvironment by producing ROS [25, 26]. Furthermore, it has been reported that administration of ROS inhibitors completely abrogated the suppressive effect of MDSCs on T cells [27]. ROS reduce T cell immune responses via inhibiting recognition between T cell receptor (TCR) and MHC-peptide complex, while adding ROS inhibitors such as catalase into the MDSCs/T cells coculture system could impair suppressive effects of MDSCs on T cell proliferation [28]. MDSCs isolated from mice lacking NOX-2 showed little or no ROS production and also failed to suppress the proliferation and IFN-γ production of T cells [29, 30]. It has been reported that MDSCs inhibited T cell activation by depleting cystine and cysteine [31], which is closely correlated with ROS production. Indeed, cystine and cysteine are essential for synthesizing the glutathione (GSH) that eliminates ROS production. In addition, scavenging of H_2O_2 with catalase induces differentiation of immature myeloid into macrophages in tumor-bearing mice, suggesting that ROS also play an important role in maintaining the undifferentiated state of MDSC [32, 33]. However, low level of ROS could activate T cells and anti-CD3 induced phosphorylation of extracellular signal-regulated ERK pathway required H_2O_2 generation [34].

TAMs are considered as critical links between inflammation and cancer development [35, 36]. ROS produced by macrophages have been reported to have immunosuppressive properties and could also be functional for induction of Tregs [37]. The ROS producing capacity by different subtypes of macrophages is discrepant. M2-type macrophages induced by M-CSF and IL-10 have a higher ROS producing capacity [38]. In contrast, CD137, a costimulatory immune checkpoint molecule, could reduce typical macrophage characteristics such as phagocytosis, oxidative burst, and CD14 expression, which could induce the differentiation of monocytes to dendritic cells (DC) and DC maturation and reduce ROS generation [39]. ROS produced by macrophages were higher than those by DCs [40] while CD137L-activated microglia induce apoptosis of oligodendrocytes dependent on ROS [41].

Apart from MDSCs and macrophages, T lymphocytes are another main source of ROS. Indeed, peripheral blood T lymphocytes from cancer patients showed an increased ROS production compared to those from healthy subjects [42]. The process of TCR activation is accompanied by ROS production, and tumor-infiltrating lymphocytes could be dysfunctional due to the ROS accumulated in the tumor microenvironment. Intracellular ROS level in T cells is tightly regulated through NOX-2, dual-substrate oxidase 1 (DUOX-1), mitochondria, and the expression of a variety of antioxidant systems, including superoxide dismutase, peroxiredoxins, and glutaredoxins coupled to metabolic status of T cells [43–45]. The major sources of ROS production in T cells are lipid metabolism, mitochondria, and NOXs [44, 46]. Mitochondria generate low amounts of ROS (superoxides) in a controlled and stimulation-dependent fashion, thereby less likely to have a direct influence on tumor cells or other surrounding cells. DUOX-1 activation generates H_2O_2 that acts in a positive feedback loop to enhance and stain further TCR signaling [45]. However, high amounts of extracellular ROS produced by an oxidative burst from macrophages or in a pathophysiological condition induce the disability of T cells [38, 47]. Interestingly, low amounts of ROS can stimulate T cell activation/proliferation [48, 49]. Tregs are key immunosuppressive cells increased in cancer patients. TGF-β secreted by Tregs activates the NOXs to produce ROS. Low level of ROS has been also shown to induce the immunoregulatory enzyme, indoleamine 2,3-dioxygenase, and enhance the function of Tregs [50]. Tregs exhibit reduced sensitivity to ROS-induced cell death, while the level of ROS determines the function of Tregs. Indeed, Tregs isolated from neutrophil cytosolic factor 1 (Ncf1) deficiency mice with a lower level of ROS were hyporeactive compared to those from wild type mice [50].

Other inflammatory cells such as neutrophils, eosinophils, and mononuclear phagocytes could produce ROS in the tumor microenvironment as well [51], thereby contributing to tumor growth and antitumor immune response.

Besides immune cells in the tumor microenvironment, tumor cells could also generate excessive ROS [42], which may be encoded from mutations of electron transport chain (ETC) mitochondria-related genes as well as the mitochondrial DNA damage. For example, a loss of p53 causes depletion of mitochondrial DNA and altered homeostasis of mitochondrial ROS [52]. ROS generated by mitochondria contribute to the initiation of nuclear of mitochondrial DNA mutations that promote neoplastic transformation [53]. ROS in cancer cells can be also driven by increased metabolism, oncogene activity, and abnormal expression of NOXs and play a doubled-edged sword role in cancer progression. The dual roles of ROS depend on their concentration [54]. On one hand, ROS could facilitate carcinogenesis and cancer progression at mild-to-moderate elevated levels. Metabolic synergy or metabolic coupling between glycolytic stromal cells (Warburg effect) and oxidative cancer cells occur in cancer and promote tumor growth, while ROS are key mediators of the stromal Warburg effect [55]. On the other hand, excessive ROS would damage cancer cells dramatically and even lead to cell death [54, 56]. Tumor cells can express increased levels of antioxidant proteins to detoxify ROS [57]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a pivotal transcription preventing oxidative stress, but aberrant activation of Nrf2 often occurs in various human cancers. Silencing Nrf2 inhibited proliferation of glioma cells via AMP-activated protein kinase- (AMPK-) activated mammalian target of rapamycin (mTOR) [58]. In contrast, capsaicin mediates bladder cancer cell death through increasing ROS production [59]. Hypoxia-inducible factor 1α (HIF-1α) can induce ROS production by acting on complexes I, II, and III of mitochondria ETC [60, 61]. Both HIF-1α and nuclear factor-κB (NF-κB) could induce the expression of MMPs to promote ROS production by regulating COX-2 in tumor cells [62]. Indeed, activities of MMP-2 and MMP-9 in tumor tissues were correlated with superoxide radicals generation rate [63]. Taken together, considering dual roles of ROS, the strategies of decreasing or increasing the level of ROS in cancer cells warrant cautious consideration for cancer treatment.

3. Regulators of ROS Production

During the process of ROS production, the level of ROS is usually regulated by many factors in the tumor microenvironment. First, there are several checkpoints restricting ROS production by the NOXs following activation of receptors by ligands such as insulin, platelet-derived growth factor, transforming growth factor, nerve growth factor, fibroblast growth factor, tumor necrosis factor-α, and epidermal growth factor [64–67]. Second, when tumor diameters reach about 200 μm, tumor tissues become hypoxic, representing a negative prognostic indicator [68, 69]. Hypoxia induces ROS production through regulating transcription Nrf2 that reduces ROS accumulation [70]. MMPs have been identified as important regulators of the activity of mitochondrial respiratory chain and intracellular ROS production [71]. Third, ROS generation was associated with cell metabolism and glucose metabolism and mitochondrial respiratory would increase ROS production [49, 72]. In addition, Calnexin expression is required for cellular NOX4 protein expression and ROS formation [73], which may regulate cell apoptosis induced by endoplasmic reticulum stress or by inositol starvation [74, 75]. Camalexin induced T-leukemia Jurkat cell apoptosis by increasing ROS concentration and activation of caspase-8 and caspase-9 [76]. Several chemotherapeutic agents, such as Chelerythrine (protein kinase C inhibitor) and Quinones, also induced tumor cells apoptosis through increasing ROS [77, 78].

Level of ROS is dynamic and regulated by antioxidant system in the body. Antioxidant mechanisms, either enzymatic (catalases, dismutases, and peroxidases) or nonenzymatic (vitamins A, C, and E and GSH), are critical to protect cells against ROS-induced damage [1]. ROS-mediated signaling can be opposed by specific antioxidants. For example, GSH, a major intracellular redox molecule that protects cells from oxidative stress [79], is essential for optimal T cell proliferation and activation, and it is synthesized by cysteine [80]. Inactivation of the extracellular superoxide dismutase (SOD) leads to accumulation of ROS in the tumor microenvironment [81]. Manganese superoxide dismutase (MnSOD) is a major antioxidative enzyme, neutralizing $O_2^{\bullet-}$ released by electron chain as a by-product of respiration. Silencing MnSOD results in increasing intracellular oxidative stress, while increasing MnSOD exerts an antitumor effect both *in vitro* and *in vivo* [82].

4. ROS Affect T Cell Activation

ROS excessive in the tumor microenvironment reduce antitumor function and proliferation of T cells and increase T cell apoptosis. ROS produced by other cells can reach T cells and cause oxidative stress which may induce T cell hyporesponsiveness in cancer patients [83]. It has been reported that exposure of T cells to high level ROS downregulates T cell activity [84]. Though exact effect of ROS on T cells function remains unclear, the balance between production and consumption of ROS is an important factor that determines the T cell apoptosis, activation, differentiation, proliferation, and function (Figure 2). Indeed, ROS at a low-concentration are essential for T cell activation, expansion, and effector function [34, 44].

TCR signaling pathways are affected differentially by physiological levels of ROS that trigger several proximal and distal signaling pathways in T cells. CD3 activation leads to rapid influx of calcium, in turn regulating ROS production [49], while Devadas shows that calcium release is essential for ROS production [34]. However, both signals are essential for T cell receptor signaling [85]. MnSOD/SOD2 participates in downregulation of TCR-induced prooxidative intracellular status. Several studies demonstrate that MnSOD regulates T cell differentiation and function through reducing activation-induced ROS production [82, 86]. Mitochondrial ROS control T cell activation by regulating IL-2 and IL-4 expression, which are determined by an oxidative signal originating from mitochondrial respiratory complex I [87]. In addition, complex I of the mitochondrial ETC is the source of activation-induced ROS formation [43]. However, mitochondrial ROS specifically derived from complex III are required for $CD4^+$ T cell activation and antigen specific T cells expansion [49]. NOX-2-deficient T cells showed enhanced Erk kinase activation and T helper type I cytokine secretion [44]. Moreover, recently it has been shown that retrograde electron flow and ROS production were important not only in T cell activation but also in aging and development of Parkinson disease [88, 89]. In the beginning of the 90th, it has been shown that ROS could activate NF-κB, while chronic exposure to ROS would inhibit NF-κB phosphorylation and activation in T cells [90, 91]. In the cytoplasm, an oxidative environment for NF-κB nuclear translocation is needed and in the nucleus a reducing environment for NF-κB DNA binding is required. Therefore, induction of low ROS levels rather enhances NF-κB (the cytosol becomes oxidizing the nucleus that is still reducing) whereas high ROS levels inhibit NF-κB (cytosol and nucleus are oxidizing) [92, 93]. Reduced ROS production in association with decreased levels of JNK and NF-κB phosphorylation has an impact on both IFN-γ and CD39 expression of $CD8^+$ T cells [94]. Reduced ROS production by antioxidants or NOX inhibitors also induced Treg hypoactivation *in vitro* [95]. Tregs can suppress cysteine release from DCs, leading to oxidation of surface thiols, thereby decreasing intracellular GSH and DNA synthesis in conventional T cells. High levels of ROS could also inhibit mTOR pathway that is crucial in T cell activation and metabolism [96]. In addition, ROS can regulate proline-rich tyrosine kinase 2 (Pyk2) phosphorylation in cytotoxic T lymphocytes (CTL) by Ca^{2+}-dependent pathways and Erk signaling [97]. For another, Granzyme B secreted by cytotoxic T cells induces proapoptotic pathways and then leads to cell death [98]. ROS produced by extramitochondria are involved in the process of Granzyme B induced cell death, most probably through activation of NOX [99]. Glutathione peroxidase 4 (Gpx4) could function as a unique antioxidant enzyme to inhibit lipid peroxidation and play a vital role in the homeostatic survival of $CD8^+$ T cells and in both $CD4^+$ and $CD8^+$ T cell expansion upon TCR triggering in response to infection by preventing membrane lipid peroxidation and ferroptosis [100].

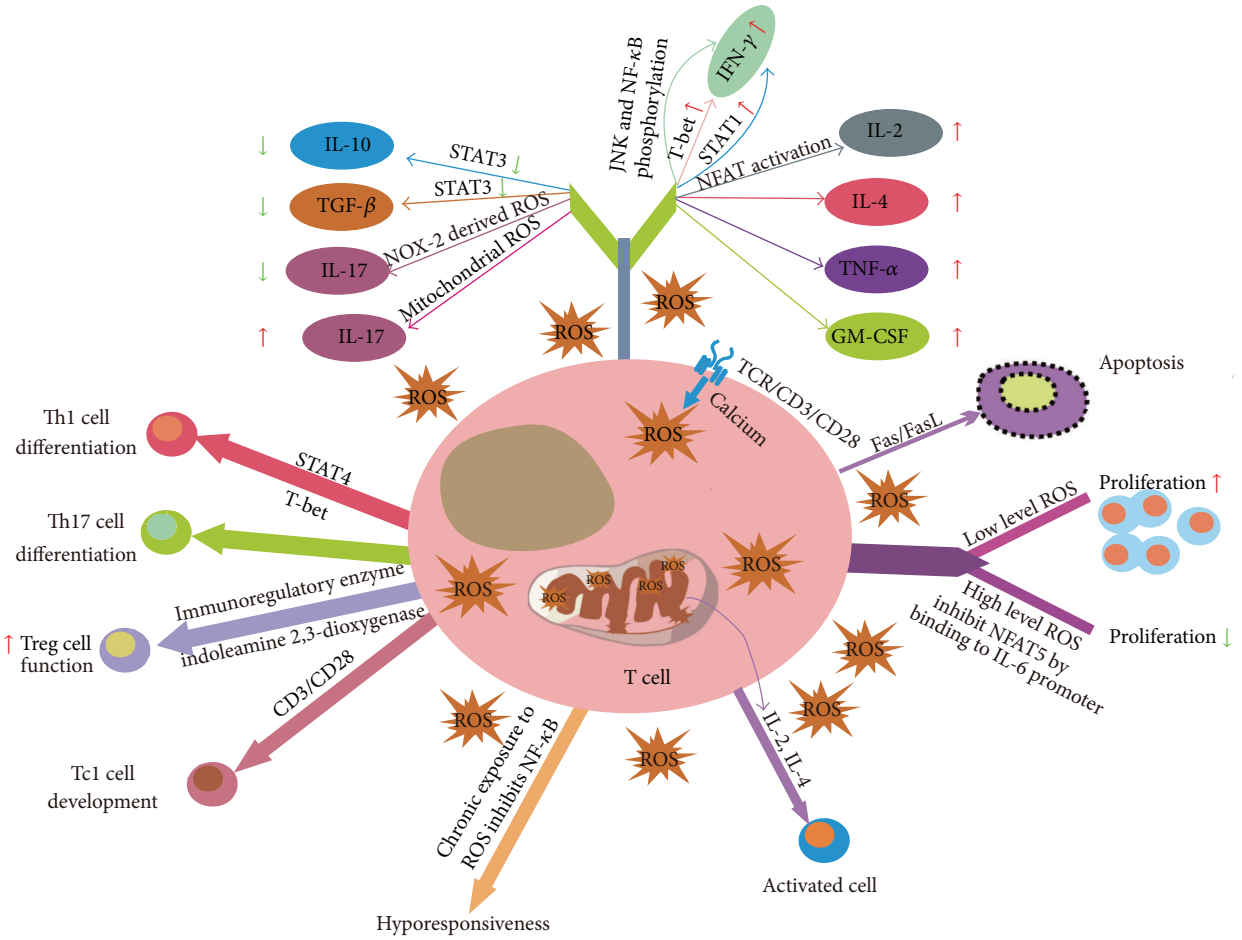

FIGURE 2: Multifaceted regulation of T cell responses by ROS. CD3 activation leads to rapid influx of calcium promoting ROS production. However, the connection between calcium and ROS production is under debate. Both signals are essential for TCR signaling. ROS trigger activation-induced cell death of T cells via Fas/FasL pathway. The low levels of mitochondrial ROS are required for T cell proliferation, while high levels of ROS inhibit NFAT5 by binding to IL-6 promoter and decrease T cell proliferation. Mitochondrial ROS are indispensable for T cell activation by regulating IL-2 and IL-4 secretion. Chronic exposure to ROS may inhibit NF-κB phosphorylation and activation, which induces T lymphocytes hyporesponsiveness. NOX-2 derived ROS increase IFN-γ production via increasing the levels of JNK and NF-κB phosphorylation, transcription factors STAT-1 and T-bet, and cytokines secretion of IL-2, IL-4, TNF-α, and GM-CSF. Further, NOX-2 derived ROS decrease phosphorylation of STAT3 and production of IL-10, TGF-β, and IL-17. Mitochondrial ROS regulate differentiation of Th17 cells and Th1 cells. Low levels of ROS induce the immunoregulatory enzyme, indoleamine 2,3-dioxygenase, and enhance the function of Tregs. NOX/ROS is a key upstream component of CD3 and CD28 signaling pathways during Tc1 cell development.

5. ROS Affect T Cell Differentiation

To explore effects of ROS on T cells differentiation, T cells with specific NOX-2 knockout or other ROS producing enzymes knockout have been studied. NOX-2 is composed of gP91^{phox} and p47^{phox}, so mice lacking either component have been identified as good models to study the role of NOX-2 derived ROS in T cells differentiation. p47^{phox} deficiency in T cells diminished the expression of transcription factors STAT1, STAT4, and T-bet and reduced the production of cytokine, such as IL-2, IL-4, IFN-γ, TNF-α, and GM-CSF [101]. In contrast, increased phosphorylation of STAT3 and production of IL-10, TGF-β, and IL-17 were further observed in p47^{phox} deficient T cells [101]. Surprisingly, CD4$^+$ T cells from gP91$^{phox-/-}$ mice displayed Th1 phenotype [102]. However, both studies have detected decreased IL-4 and increased IL-17 production in NOX-2-deficient cells, suggesting a possible role of NOX complex in Th17 cell differentiation. Indeed, specific mitochondria ROS inhibitors such as N-acetylcysteine and mitoquinone reduced production of Th17 cells [103], whereas mitochondrial ROS were historically thought to be primarily cytotoxic by directly damaging DNA, lipids, and proteins [104]. Moreover, gene IEX-1 deficiency facilitated Th17 cell differentiation during early responses, which was mediated by increased formation of ROS at mitochondria following T cell activation [103].

6. ROS Affect T cell Apoptosis

Mitochondrial ROS are indispensable for T cell activation-induced expression of Fas ligand (FasL) that mediates activation-induced cell death (AICD) [43, 82]. Different

sources of ROS are involved in AICD of T cells. TCR-stimulated upregulation of FasL and subsequent AICD was dependent upon superoxide anion, but independent of H_2O_2 [34]. ROS induce the expression of FasL that further activates NOX-2, which participates in the apoptotic program via ROS-mediated AKT activation and MEK inhibition [105]. Programmed death-1 (PD-1) is described initially as a marker of apoptosis and is considered as a checkpoint that controls T cell function. PD-1 blockade has been recently approved to treat patients with advanced-stage cancers by enhancing antitumor T cell immunity [106]. As the expression level of PD-1 is correlated with production of cellular ROS and oxidative metabolism [107], it would be interesting to explore potential strategies of combining ROS scavenger with PD-1 signaling blockade for rapid clinical translation.

The susceptibility of human T cells to H_2O_2-induced apoptosis strongly varies among T cell subsets. T cells resistance to exogenous H_2O_2 decreases in the following order: effector T cells > regulatory T cells > naive T cells > memory T cells [108]. $CD8^+$ effector memory T cells are more sensitive to ROS compared with other T cells types [109]. It is likely that effector T cells are most insensitive to ROS-mediated death. Several studies have shown that GSH plays essential roles in increasing T cell function and proliferation [15, 110], while ROS scavenger could reduce ROS-induced apoptosis of naive and memory cells. Furthermore, a correlation between intracellular GSH depletion and progression of apoptosis has been confirmed in several studies [111–113]. Additionally, high GSH levels are associated with an apoptotic resistant phenotype in different cells. In general, TCR-stimulated ROS generation in T cells serves to regulate a proapoptotic pathway (FasL-mediated) and a proliferative pathway (ERK-mediated) that are critical for T cell function and survival.

Given importance of nuclear factor of activated T cell 5 (NFAT5) in T cell proliferation and survival [114], inhibition of binding of NFAT5 to IL-6 promoter by ROS may participate in the regulation of T cell responses. In addition, oxidative stress is a central regulator of HMGB1 translocation, release, and activity [115]. For example, mitochondrial ROS oxidation releases high mobility group box 1 (HMGB1) during apoptosis, while both intracellular and extracellular HMGB1 play pivotal roles in regulating T cell immune responses [116].

7. Conclusions and Perspectives

ROS produced mainly by tumor cells and immunosuppressive cells in the tumor microenvironment may determine the activation, proliferation, differentiation, and apoptosis of antitumor T cells. Considering the ROS-mediated immunosuppressive mechanisms, an important implication of therapeutic strategy targeting ROS is using antioxidant agents or supplements which may regulate antitumor T cell responses. Specifically, T cell-based therapy combined with ROS scavenger would improve clinical efficacy by enhancing expansion and function of antitumor T cells. Despite remarkable progress in recent years, the mechanism for the roles of ROS in T cell biology still remains unclear. Development of more effective strategies combining ROS manipulation and T cell-based therapy warrants further investigations particularly for the treatment of patients with advanced cancer.

Competing Interests

The authors have no competing interests to disclose.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (no. 81171986 and no. 81271815), Research Grant from the Ministry of Public Health (no. 201501004), the Basic and Advanced Technology Research Foundation from Science and Technology Department of Henan Province (no. 112300410153 and no. 122300410155), Funds for Creative Research Team of Henan Province, Creative Research Team of Higher Education of Henan Province, and International Cooperative Research of Henan Province.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5190314, 9 pages
http://dx.doi.org/10.1155/2016/5190314

Research Article

Hyperglycemia Promotes the Epithelial-Mesenchymal Transition of Pancreatic Cancer via Hydrogen Peroxide

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Received 14 March 2016; Revised 15 April 2016; Accepted 21 April 2016

Academic Editor: Alexandr V. Bazhin

Diabetes mellitus (DM) and pancreatic cancer are intimately related, as approximately 85% of patients diagnosed with pancreatic cancer have impaired glucose tolerance or even DM. Our previous studies have indicated that high glucose could promote the invasive and migratory abilities of pancreatic cancer cells. We therefore explored the underlying mechanism that hyperglycemia modulates the metastatic potential of pancreatic cancer. Our data showed that streptozotocin- (STZ-) treated diabetic nude mice exhibit larger tumor size than that of the euglycemic mice. The number of nude mice that develop liver metastasis or ascites is much more in the STZ-treated group than that in the euglycemic group. Hyperglycemic mice contain a higher plasma H_2O_2-level than that from euglycemic mice. The injection of polyethylene glycol-conjugated catalase (PEG-CAT), an H_2O_2 scavenger, may reverse hyperglycemia-induced tumor metastasis. In addition, hyperglycemia could also modulate the expression of epithelial-mesenchymal transition- (EMT-) related factors in pancreatic tumor tissues, as the E-cadherin level is decreased and the expression of mesenchymal markers N-cadherin and vimentin as well as transcription factor snail is strongly increased. The injection of PEG-CAT could also reverse hyperglycemia-induced EMT. These results suggest that the association between hyperglycemia and poor prognosis of pancreatic cancer can be attributed to the alterations of EMT through the production of hydrogen peroxide.

1. Introduction

As the fourth leading cause of cancer death worldwide, pancreatic cancer (PC) is an aggressive and intractable malignant disease due to the lack of early symptoms, poor prognosis, short survival, and resistance to therapy [1]. Approximately 75% of PC patients die within 1 year of diagnosis and only 5% or less survive for 5 years [2]. It has been projected that PC will become the leading cause of cancer-related deaths in the USA by 2050 [3]. In China, it has been estimated that 90,100 subjects will be newly diagnosed with PC and will account for 79,400 cancer-related death in 2015 [4]. Due to the fact that almost 80% of PC patients have locally deteriorated or metastatic disease, they are not appropriate for resection in the early stage of tumor development [5]. It is important to highlight cellular mechanisms of the etiological and risk factors to this disease which might lead us to find more effective therapeutic strategies.

Diabetes mellitus (DM), a major worldwide public health problem, is associated with certain site-specific cancers, including liver, biliary tract, pancreatic, and colorectal cancer [6–9]. In recent years, DM has been postulated to be both an independent risk factor and a consequence for PC and up to 80% of PC patients have pathologic glucose tolerance or DM test at diagnosis [10]. Our previous *in vitro* studies showed that high glucose (HG) can be regarded as an accelerator to increase cell proliferation through enhanced epidermal growth factor (EGF)/EGFR signaling [11]. We have also proven that hyperglycemia may worsen the prognosis of PC by enhancing their migratory and invasive ability through the production of hydrogen peroxide (H_2O_2) [12], which might be modulated by the expression of superoxide dismutase (SOD) through the activation of the ERK and p38 MAPK signaling pathways [13]. In addition, we also demonstrated that DM enhances perineural invasion in PC patients and aggravates a poor prognosis [14]. The inner mechanism between PC metastasis and DM should be deeply evaluated.

Distant metastasis, considered as the pivotal step in solid tumor progression, is responsible for approximately 90% of cancer-related deaths [15]. The pathogenesis of cancer

metastasis is complex and not fully understood. Epithelial-mesenchymal transition (EMT), which is originally established during embryogenesis, has been intimately related with cancer metastasis by allowing a polarized epithelial cell to assume a mesenchymal cell phenotype and gaining enhanced migratory and invasive capacity [10]. A typical symbol of EMT includes a striking decline in the cell-cell adhesion molecule E-cadherin expression and gain of mesenchymal markers, such as vimentin and N-cadherin, culminating in cell morphology change as well as enhanced cell motility [16]. Recently, accumulating data and studies have started to indicate the relationship between hyperglycemia and EMT, especially on diabetic renal injury [17, 18] and peritoneal dialysis [19, 20]. Our recent studies proved that H_2O_2 production can promote EMT in PC, leading to increased motility and invasion via activation of ERK signaling pathway [21]. However, whether hyperglycemic condition could influence EMT in PC has not been elucidated.

In the current study, we investigated the production of H_2O_2 in both STZ-treated diabetic nude mice and euglycemic nude mice. We also tested the hypothesis that H_2O_2 mediates hyperglycemia-induced EMT and regulates the metastatic activity of PC. Our findings may provide new insight on the relationship between DM and PC and reveal a novel therapeutic strategy for PC patients who suffer from diabetes.

2. Materials and Methods

2.1. Cell Culture and Reagents. The human PC cell lines Panc-1, obtained from the American Type Culture Collection (Manassas, VA, USA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% dialyzed heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 95% air/5% CO_2 humidified atmosphere at 37°C. DMEM and FBS were purchased from HyClone (Logan, UT, USA). Streptozotocin (STZ) and CAT derivative conjugated with polyethylene glycol (PEG-CAT) were acquired from Sigma Aldrich (St. Louis, MO, USA). The primary antibodies against E-cadherin, N-cadherin, vimentin, snail, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The BCA assay kit was purchased from Pierce (Rockford, IL, USA). Other reagents were purchased from common commercial sources. All drug solutions were freshly prepared on the day of testing.

2.2. Diabetes Mouse Model. BALB/c athymic nude mice (male, 5 weeks old) were purchased from Shanghai Experimental Animal Center (Chinese Academy of Sciences, China). Animal care and experiments were carried out in accordance with guidelines of the Xi'an Jiaotong University. BALB/c athymic nude mice were grouped into euglycemia, hyperglycemia, euglycemia + PEG-CAT, and hyperglycemia + PEG-CAT groups ($n = 6$), of which hyperglycemia mice received an intraperitoneal injection of STZ dissolved in sodium citrate buffer (pH 4.5) at a dose of 175 mg/kg body weight. Blood glucose levels were determined with an ACCU-CHEK Active meter (Hoffmann-La Roche, Basel, Switzerland).

2.3. Orthotopic Tumor Model. At 2 weeks after STZ injection, Panc-1 cells (1×10^8) were injected in a total volume of 50 μL PBS into the body of the pancreas. 3 days later, a dose of 1000 units/d PEG-CAT was intraperitoneally injected into nude mice (euglycemia + PEG-CAT and hyperglycemia + PEG-CAT groups). The mice were sacrificed after 8 weeks and the tumors, liver, spleen, pancreas, and blood were collected and analyzed. Tumor volumes were determined by using the formula width^2 $\times$ length $\times$ 0.5.

2.4. Hydrogen Peroxide Assay. The level of mice plasma H_2O_2 was measured using a H_2O_2 assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. In this kit, the ferrous ions Fe^{2+} were oxidized to ferric ions Fe^{3+} by H_2O_2. The ferric ions further formed a complex with the indicator dye xylenol orange and produced a visible purple-colored complex that could then be measured using a microplate reader at a wavelength of 560–590 nm (Bio-Rad, CA, USA) [22].

2.5. Real-Time Quantitative PCR (qRT-PCR). Total RNA was extracted from the orthotopic tumor of pancreas using the Fastgen200 RNA isolation system (Fastgen, Shanghai, China) according to the manufacturer's protocol. Total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The primer sequences were as follows:

E-Cadherin-F: 5′-ATT CTG ATT CTG CTG CTC TTG-3′.

E-Cadherin-R: 5′-AGT CCT GGT CCT CTT CTC C-3′.

N-Cadherin-F: 5′-ATG GTG TAT GCC GTG AGA AG-3′.

N-Cadherin-R: 5′-TGT GCT TAC TGA ATT GTC TTG G-3′.

Vimentin-F: 5′-AAT GAC CGC TTC GCC AAC-3′.

Vimentin-R: 5′-CCG CAT CTC CTCCTC GTA G-3′.

Snail-F: 5′-CTT CTC CTC TAC TTC AGT CTC TTC-3′.

Snail-R: 5′-CGT GTG GCT TCG GAT GTG-3′.

β-actin-F: 5′-GAC TTA GTT GCG TTA CAC CCT TTC T-3′.

β-actin-R: 5′-GAA CGG TGA AGG TGA CAG CAG T-3′.

After each qRT-PCR, a dissociation curve analysis was conducted. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method reported previously [23]. Each measurement was carried out in triplicate.

2.6. Western Blotting. After being electrophoretically resolved on a denaturing SDS-polyacrylamide gel, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were initially blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for

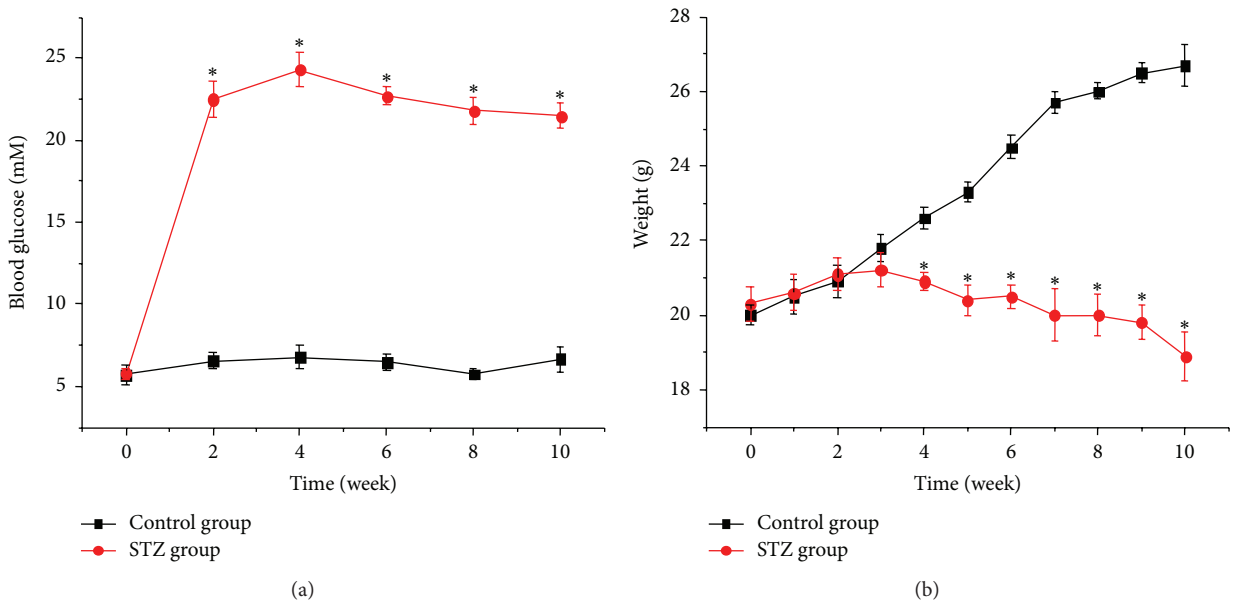

Figure 1: Effect of STZ on blood glucose and weight in nude mice. (a) Blood glucose in STZ-treated mice ($n = 12$). (b) Body weight in STZ-treated mice ($n = 12$). $*$ refers to $P < 0.05$ as compared with control group.

2 h and then probed with antibodies against E-cadherin, N-cadherin, vimentin, snail, or β-actin as loading control. After coincubation with the primary antibodies at 4°C overnight, membranes were incubated with the secondary antibody for 2 h at room temperature. The results were visualized using the ECL Western blotting substrate and photographed by GeneBox (SynGene).

2.7. Immunohistochemistry. Formalin fixed and paraffin embedded orthotopic pancreatic tumor tissue samples were used for the immunohistochemistry test. In brief, the tissue sections were incubated with primary antibodies (anti-E-cadherin, anti-N-cadherin, anti-vimentin, and anti-snail, 1 : 50) overnight at 4°C and incubated with the appropriate biotinylated secondary antibody for 30 min at room temperature. After rinsing, the results were visualized using diaminobenzidine (DAB) and the slides were counterstained with hematoxylin. The densitometry analysis of immunohistochemical staining was performed using the Image-Pro Plus 6.0 software.

2.8. Statistical Analysis. Statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, USA). Data were presented as the means ± SEM of three replicate assays. Differences between the groups were assessed by analysis of Chi-square test and analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$. All experiments were repeated independently at least three times.

3. Results

3.1. STZ Treatment Increases the Fasting Blood Glucose Levels and Decreases the Body Weight of the Nude Mice. To determine the efficacy of different drugs against transplantation-established human tumor xenografts in the athymic nude mice, we used an orthotopic tumor model. STZ is a chemical which is commonly used to induce experimental diabetes in animals [24]. The characteristics of the STZ-treated nude mice used in this study were summarized in Figure 1. The fasting blood glucose levels were significantly increased from 2 weeks to 4 weeks and keep a high level till 10 weeks after STZ injection (Figure 1(a)). The body weight of the nude mice were reduced at 4 weeks after STZ injection (Figure 1(b)).

3.2. Hyperglycemia Enhances the Production of H_2O_2 in Mice Plasma. Hyperglycemic condition has been shown to induce the overproduction of reactive oxygen species (ROS), which consists of a number of chemically reactive molecules derived from oxygen, including H_2O_2 [25]. Our previous *in vitro* study also proved that H_2O_2 is able to mediate high glucose-induced invasive activity via ERK and p38 MAPK in human PC cells [13]. In this study, we tested the level of H_2O_2 in mice plasma before the mice were sacrificed. As shown in Figure 2, hyperglycemic mice contained a higher plasma H_2O_2 level than that from euglycemic mice. PEG-CAT could significantly reduce the blood H_2O_2 level of STZ-injected mice.

3.3. Hyperglycemia Induces Pancreatic Tumor Growth in Nude Mice. Our previous study has proven that high glucose (25, 50 mM) could significantly increase the proliferation of PC cells compared with low glucose (5.5 mM) via the induction of EGF expression and transactivation of EGFR. The stimulating effect on cell proliferation in PC may be through the effect of accelerating cell cycle progression [11]. Here we found that the tumor volume and weight were increased in hyperglycemic mice than those in euglycemic mice. To evaluate whether the promotion of tumor growth is associated with the production of H_2O_2, mice were treated with PEG-CAT. As shown in Figure 3, the tumor volume and

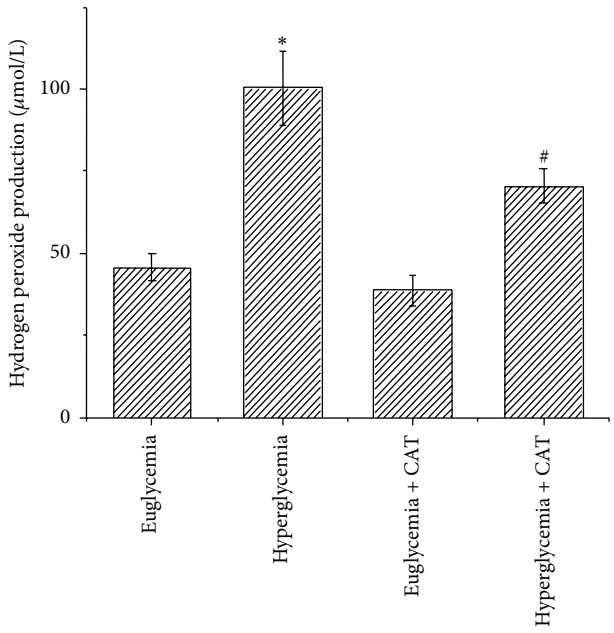

Figure 2: Effect of hyperglycemia on H_2O_2 production in mice plasma. The levels of H_2O_2 in mice plasma were tested before the mice were sacrificed. $^*P < 0.05$ as compared with euglycemia group; $^\#P < 0.05$ as compared with hyperglycemia group.

Table 1: The number of mice that develop liver metastasis, spleen metastasis, or ascites.

Groups (number)	Ascites	Liver metastasis	Spleen metastasis
Euglycemia (6)	1	0	1
Hyperglycemia (6)	5*	4*	2
Euglycemia + PEG-CAT (6)	0	0	0
Hyperglycemia + PEG-CAT (6)	2	1	0

$^*P < 0.05$ as compared with euglycemia group.

weight of hyperglycemic mice did not change after PEG-CAT injection.

3.4. Hyperglycemia Promotes Ascites Production and Liver Metastasis via H_2O_2 Production in PC. To determine whether hyperglycemic condition and H_2O_2 were involved in PC metastasis, equal numbers (1×10^8) of Panc-1 cells were injected into the body of mice pancreas (both euglycemic mice and hyperglycemic mice). 3 days later, a dose of 1000 units/d PEG-CAT was intraperitoneally injected into nude mice. Eight weeks after injection of the cells, mice were sacrificed and the metastatic tumors were recorded (Figure 4). As described in Table 1, only one out of six euglycemic animals generated ascites, whereas five out of six hyperglycemic mice generated ascites. Two mice in the hyperglycemia + PEG-CAT group produced ascites. In addition, none of the euglycemic mice developed visible liver metastasis, whereas four out of six hyperglycemic mice developed liver metastasis. After injected PEG-CAT, only one hyperglycemic mouse developed liver metastasis. Taken together, these results show that the number of mice that develop liver metastasis or ascites is much more in the STZ-treated group than that in the euglycemic group. PEG-CAT injection might reverse these effects. The hyperglycemia-induced level of H_2O_2 might be involved in the acceleration of tumor metastasis.

3.5. Hyperglycemia Induces EMT via H_2O_2 Production in PC. To further confirm whether hyperglycemia could induce EMT in PC, we examined the expression of EMT markers in the tumor tissue using immunohistochemical staining. As illustrated in Figure 5, the expression of E-cadherin was located in cell membrane, whereas N-cadherin, vimentin, and snail were mainly localized in cytoplasm. The E-cadherin staining of tumor cells was stronger in the euglycemia group than that in the hyperglycemia group, indicating that hyperglycemia was able to decrease the expression of E-cadherin. The percentage of cancer area with positive E-cadherin staining cancer cells was higher in the euglycemia group than that in the hyperglycemia group. In contrast, the N-cadherin, vimentin, and snail staining in the cytoplasm of the cancer cells was significantly stronger in the hyperglycemia group than that in the euglycemia group.

To determine whether hyperglycemia-induced EMT was H_2O_2-related, we analyzed the expression of E-cadherin, N-cadherin, vimentin, and snail using Western blotting analysis. As shown in Figures 6(a) and 6(b), the protein level of E-cadherin in hyperglycemia group was lower than that in the euglycemia group. The expression of mesenchymal markers N-cadherin and vimentin as well as transcription factor snail was stronger in hyperglycemia group. PEG-CAT injection could reverse these hyperglycemia-induced effects. We next evaluate the effects of hyperglycemia and PEG-CAT on the expression of E-cadherin, N-cadherin, vimentin, and snail at mRNA level by qRT-PCR. As shown in Figure 6(c), PEG-CAT counterbalanced hyperglycemia-induced EMT-related factors at the mRNA level and the trend was consistent with the protein results. Taken together, our results demonstrate that hyperglycemia could induce EMT progression and facilitate tumor metastasis via the production of H_2O_2 in PC.

4. Discussion

As one of the most lethal malignant diseases, PC is characterized by early invasion and metastasis, which partially account for a compromised therapeutic effect and poor outcome [26]. In recent years, although the largest improvements in survival have been made for a number of cancers, PC still shows the least improvement [1]. Therefore, the exploration of risk factors and metastatic mechanisms might lead us to find more effective therapeutic strategies for PC.

DM, a common metabolic disorder characterized by hyperglycemia, has been postulated to be both an independent risk factor and a consequence for PC in recent years [27]. A meta-analysis of 6 case-control studies and 3 cohort studies showed that a 2-fold higher risk of PC was observed in type-1 DM patients compared with individuals without DM [28]. Another meta-analysis from three large case-control studies revealed a 1.8-fold increase in risk of pancreatic cancer associated with type-2 DM [11]. In addition,

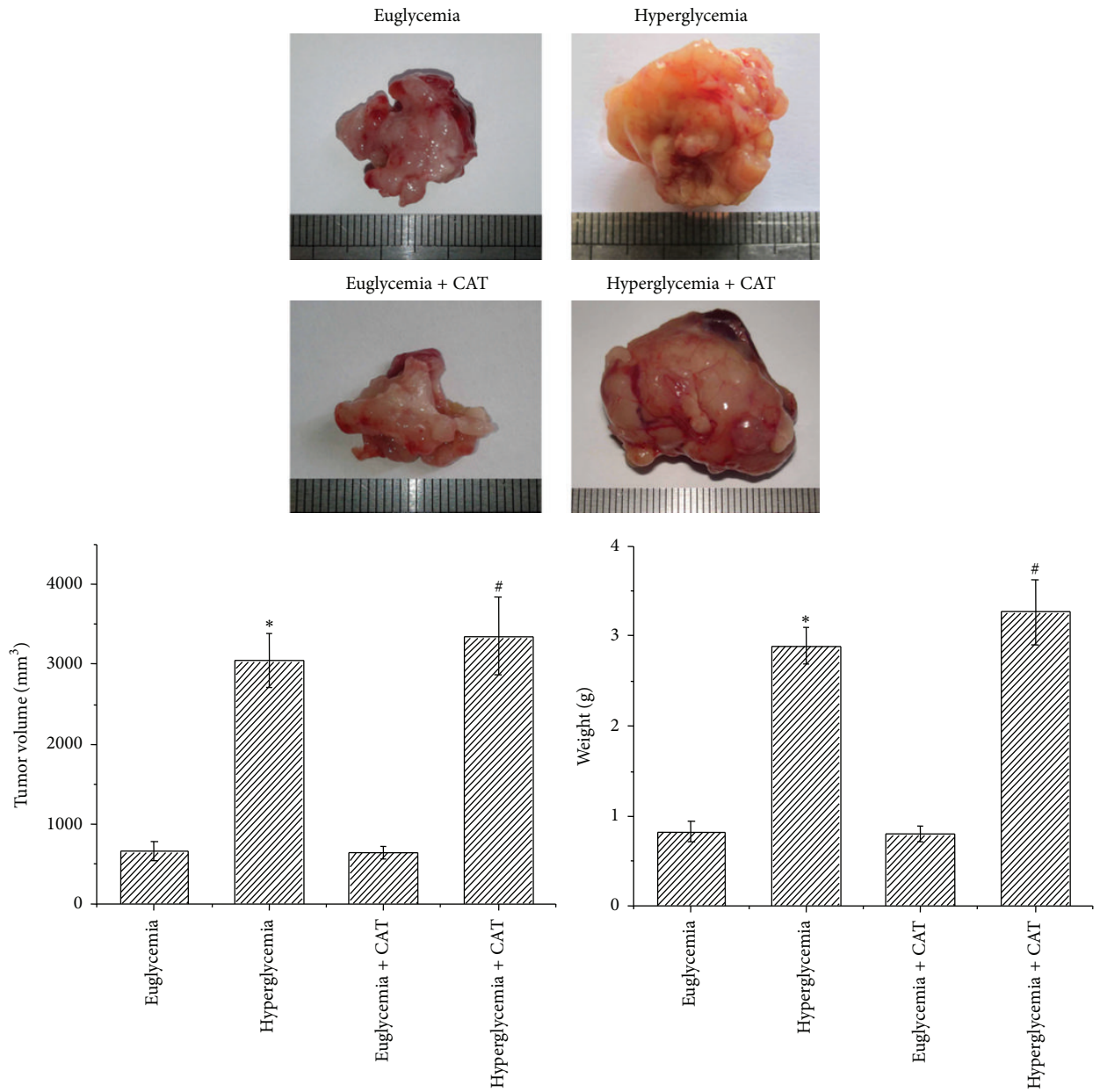

FIGURE 3: Effect of hyperglycemia on tumor growth in nude mice. Macroscopic appearance of solid tumors as well as tumor volumes and weights were tested after mice were sacrificed. $^{*}P < 0.05$ as compared with euglycemia group; $^{\#}P < 0.05$ as compared with euglycemia + CAT group.

a recent meta-analysis of 3 case-control studies and 10 cohort studies showed that using of metformin appeared to be associated with a reduced risk of pancreatic cancer in patients with type-2 DM [29]. In Chinese Han people, a moderate increased risk of pancreatic cancer was discovered among cases with long-standing diabetes, with an AOR of 2.11 (1.51–2.94), while in the cases with new-onset DM (i.e., less than 24 months in duration), the AOR is 4.43 (3.44–5.72) compared to those without DM [30]. We have proven that glucose concentrations could alter the expression of glial cell line-derived neurotrophic factor and its tyrosine kinase receptor RET in a concentration-dependent manner, correspondingly with the alterations of cell proliferation [31]. Our previous study has also shown that high glucose may worsen the prognosis of pancreatic cancer by enhancing their migratory and invasive ability through SOD-induced H_2O_2 production via the activation of the ERK and p38 MAPK signaling pathways [12, 13]. In addition, the invasive ability of both the BxPC-3 and Panc-1 cells was strongly enhanced in the DM renal capsule xenograft model and this increase could be suppressed by PEG-CAT treatment [13]. In the current study, we showed that DM was able to promote liver metastasis or ascites production in the orthotopic tumor model.

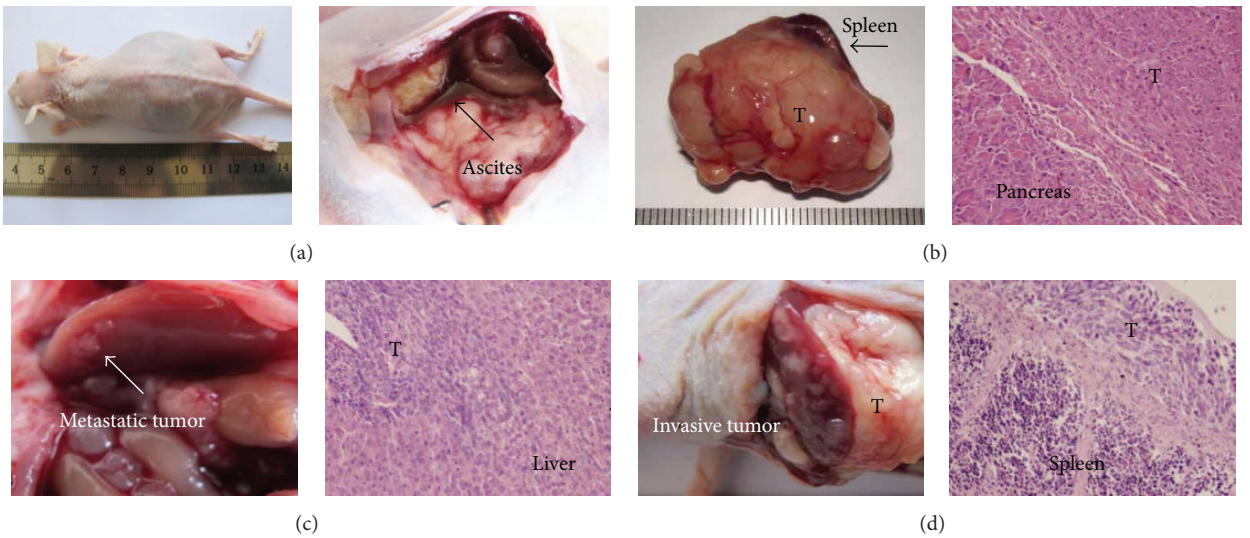

FIGURE 4: The metastasis of pancreatic tumor in nude mice. Metastatic Panc-1 tumors were analyzed by hematoxylin-eosin staining. (a) Ascites generation; (b) pancreatic tumor; (c) liver metastatic tumor; (d) spleen metastatic tumor. Original magnification ×400.

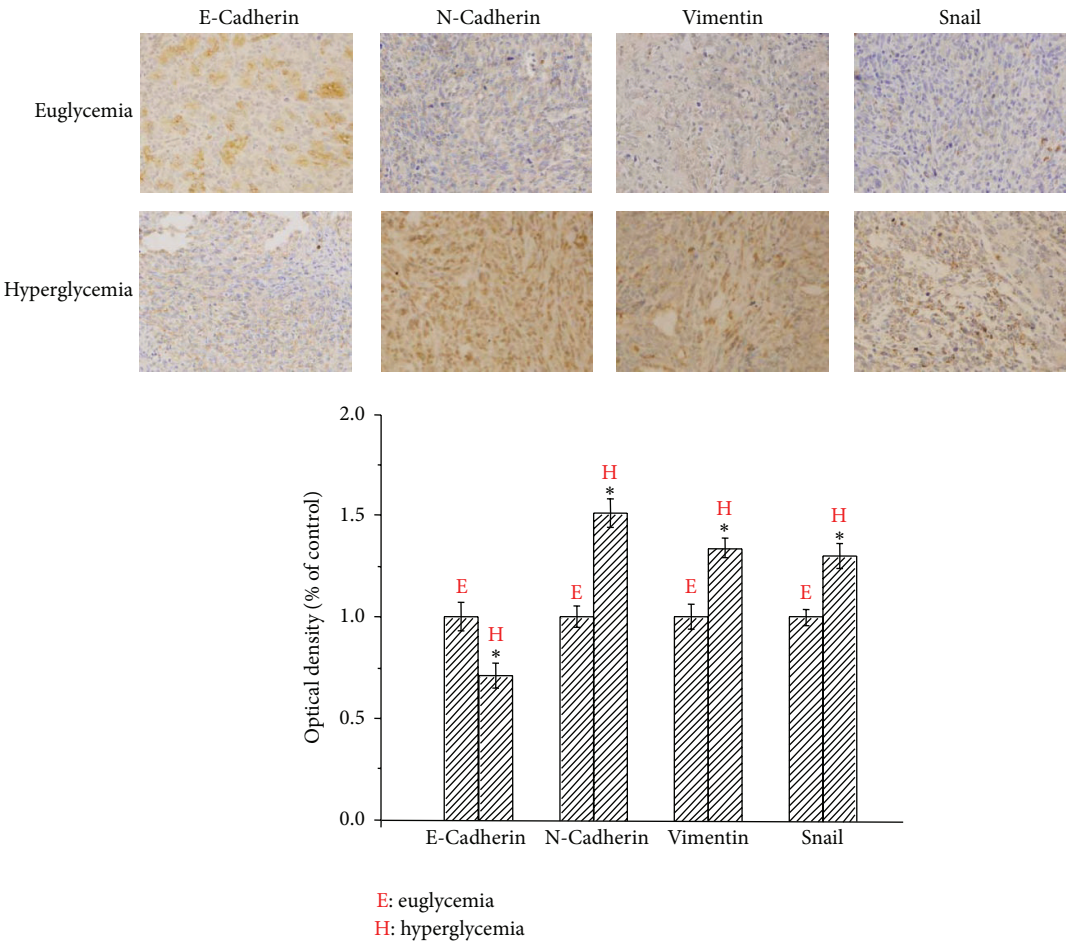

FIGURE 5: Effect of hyperglycemia on the expression of E-cadherin, N-cadherin, vimentin, and snail in nude mice. Immunohistochemistry was performed to compare the expression of E-cadherin, N-cadherin, vimentin, and snail in the orthotopic nude mice between euglycemia group and hyperglycemia group. $^{*}P < 0.05$ as compared with the euglycemia group. Original magnification ×400.

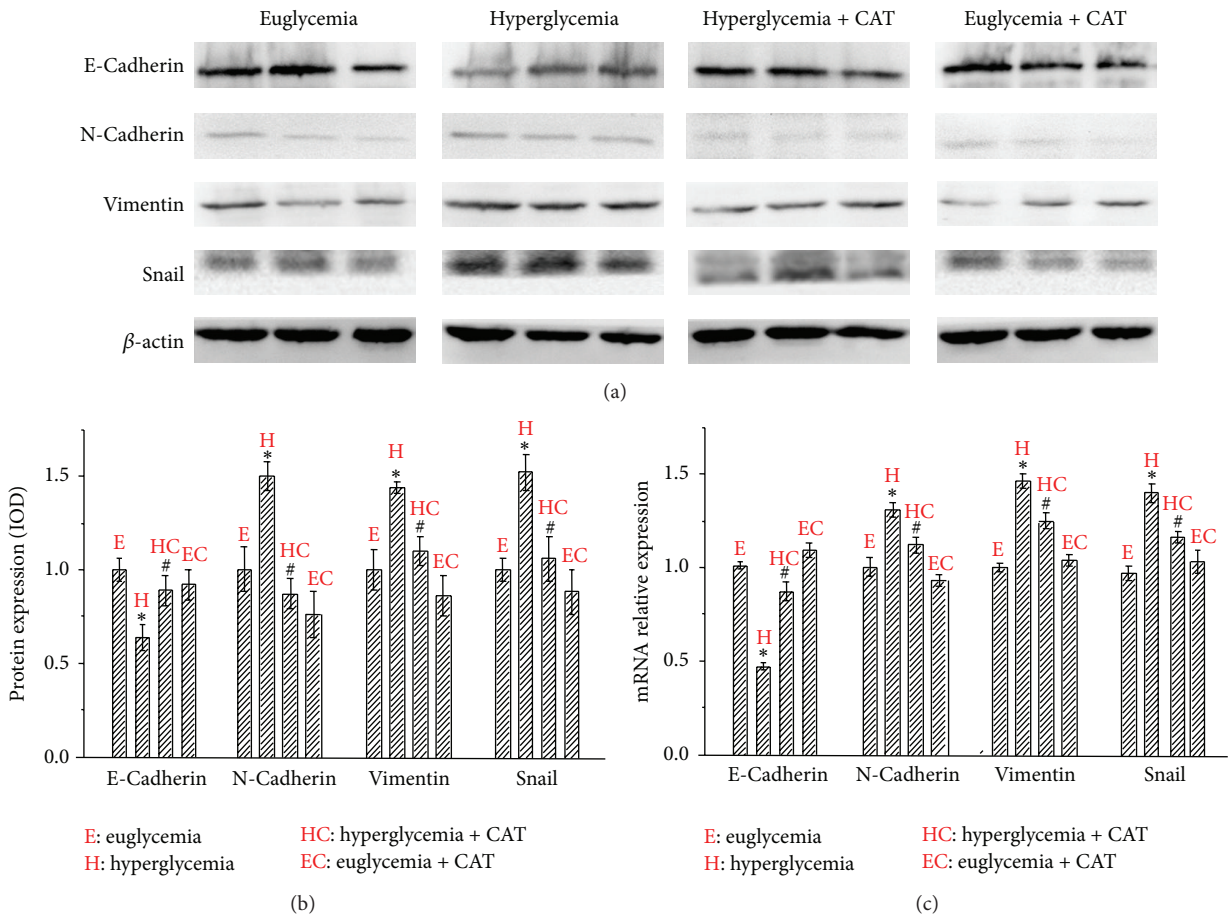

Figure 6: Effect of the hyperglycemia/H_2O_2 axis on EMT in nude mice. (a) The protein levels of EMT-related factors in pancreatic tumor tissues with different serum glucose levels were analyzed using Western blotting. (b) The statistical diagram of Western blotting analysis. (c) The mRNA levels of EMT-related markers in pancreatic tumor tissues with different serum glucose levels were analyzed using qRT-PCR. $^*P < 0.05$ as compared with the euglycemia group. $^\#P < 0.05$ as compared with hyperglycemia group.

EMT, a pivotal step in tumor metastasis, contains three essential processes: first, alterations of cell-cell and cell-extracellular matrix (ECM) interactions occur releasing the epithelial cells from the surrounding tissue. Then the cytoskeleton is reorganized so that the cells can gain the ability to move through ECM. After that, a new transcriptional program is induced to acquire morphological and functional characteristics of mesenchymal-like cells and gaining enhanced migratory and invasive capacity [10]. Our previous study has proven that SOD-induced H_2O_2 production can promote EMT in pancreatic cancer cells, leading to increased motility and invasion via activation of ERK signaling pathway [21]. The relationship between hyperglycemia and EMT has been revealed especially on diabetic renal injury and peritoneal dialysis. EMT contributes to the accumulation of matrix proteins in kidneys, in which renal tubular epithelial cells play an important role in progressive renal fibrosis. Kang et al. [32] revealed that high glucose could induce renal EMT through increasing expression of the mesenchymal markers vimentin, α-smooth muscle actin, and fibroblast-specific protein-1 in human renal proximal tubular epithelial cells and diabetic mice. A recent research also showed that HG could induce EMT in human lung adenocarcinoma epithelial A549 cells, as demonstrated by the secretion of TGF-β, cell morphology changes, the emergence of mesenchymal markers, and increased cellular motility [33]. In the current study, we showed that a single injection of STZ could lead to significant increase in fasting blood glucose in nude mouse. Hyperglycemic condition could promote tumor metastasis to liver and ascites production which might be attributed to the occurrence of EMT.

ROS generated by the mitochondrial respiratory chain consists of a number of chemically reactive molecules derived from oxygen, such as superoxide anion and H_2O_2. Accumulating evidence indicates that the intracellular redox state plays an important role in cellular signaling transduction and regulates multiple events, including tumor metastasis [34]. On one hand, an excessive amount of ROS production can kill cancer cells, whereas moderate concentrations of ROS can stimulate tumor progression by promoting cell proliferation, survival, invasion, and metastasis [35]. Our previous study has summarized that hyperglycemia is able to promote the invasive and migratory activity of BxPC-3 and Panc-1 cells via ROS production [12]. In order to confirm

whether hyperglycemia-induced EMT is regulated by H_2O_2, we treated hyperglycemic mice with PEG-CAT that could eliminate H_2O_2. Our results confirm that hyperglycemia-induced H_2O_2 influences the metastasis ability via EMT in the pancreatic cancer. Recently, Ikemura et al. [36] proved that there were greater and more numerous tumor metastatic colonies in the lung and liver of the STZ-treated mice, and injections of PEG-CAT were effective in inhibiting tumor metastasis which was consistent with our results.

5. Conclusion

In conclusion, our results indicate that hyperglycemia is correlated with tumor size, liver metastasis, or ascites formation of pancreatic cancer. The hyperglycemia-induced enhanced metastasis ability might be attributed to the occurrence of EMT via the production of H_2O_2. Our findings may provide new insight on the relationship between DM and PC. Managing hyperglycemia/H_2O_2 axis might be a novel strategy for the treatment of this severe malignancy. Our findings warrant further investigation of this hypothesis.

Competing Interests

The authors declare that there are no competing interests in this study.

Authors' Contributions

Wei Li and Lun Zhang contributed equally to this work.

Acknowledgments

This study was supported by grant from the National Natural Science Foundation of China (no. 81301846).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1908164, 12 pages
http://dx.doi.org/10.1155/2016/1908164

Review Article

Molecular and Cellular Effects of Hydrogen Peroxide on Human Lung Cancer Cells: *Potential Therapeutic Implications*

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Received 15 March 2016; Accepted 10 May 2016

Academic Editor: Alexandr V. Bazhin

Lung cancer has a very high mortality-to-incidence ratio, representing one of the main causes of cancer mortality worldwide. Therefore, new treatment strategies are urgently needed. Several diseases including lung cancer have been associated with the action of reactive oxygen species (ROS) from which hydrogen peroxide (H_2O_2) is one of the most studied. Despite the fact that H_2O_2 may have opposite effects on cell proliferation depending on the concentration and cell type, it triggers several antiproliferative responses. H_2O_2 produces both nuclear and mitochondrial DNA lesions, increases the expression of cell adhesion molecules, and increases p53 activity and other transcription factors orchestrating cancer cell death. In addition, H_2O_2 facilitates the endocytosis of oligonucleotides, affects membrane proteins, induces calcium release, and decreases cancer cell migration and invasion. Furthermore, the MAPK pathway and the expression of genes related to inflammation including interleukins, TNF-α, and NF-κB are also affected by H_2O_2. Herein, we will summarize the main effects of hydrogen peroxide on human lung cancer leading to suggesting it as a potential therapeutic tool to fight this disease. Because of the multimechanistic nature of this molecule, novel therapeutic approaches for lung cancer based on the use of H_2O_2 may help to decrease the mortality from this malignancy.

1. Introduction

Lung cancer is one of the main causes of cancer deaths worldwide [1]. Lung cancer can be divided into two major groups according to the pathological classification: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC is a cancer of epithelial origin that comprises several histological subtypes that differ in their cytology, embryonic origin, anatomical location, and oncogene expression [2]. The most common subtypes of NSCLC are adenocarcinoma (40% of all forms of lung cancer), squamous cell carcinoma (25 to 30%), and large-cell carcinoma (10 to 15%) [3]. More than 80% of NSCLC display in many cases high metastatic potential and drug resistance, resulting in poor prognosis even with an early diagnosis [4]. Therefore, new treatment strategies are urgently needed.

Reactive oxygen species (ROS) are radicals, molecules, or ions with a sole unpaired electron in the outermost shell of electrons [5]. They are well known cytotoxic agents involved in the etiology of several human diseases including cancer.

Accordingly, the expression of ROS detoxifying antioxidant proteins is altered in cancer cells in comparison to normal cells. For instance, thioredoxin reductase, thioredoxin, peroxiredoxin, glutathione S-transferase pi 1, glucose-6-phosphate dehydrogenase, and apurinic/apyrimidinic endonuclease 1/ref-1 (APE1/ref-1) have been found to be increased, while glutamate-cysteine ligase and γ-glutamyltransferase have been found to be decreased in lung cancer cells [6, 7]. APE1/ref-1 is a key enzyme in base excision repair and in the transcriptional modulation against oxidative stress. APE1/ref-1 is mainly localized in the nucleus of nontumor regions of the lung cancer tissue samples. However, nuclear and cytoplasmic expression of APE1/ref-1 is markedly upregulated in NSCLC, and the treatment of H460 lung cancer cells with hydrogen peroxide increases APE1/ref-1 expression [7].

ROS are considered potential carcinogens, since they play a role in mutagenesis, cancer promotion, and progression [8]. However, ROS also have anticancer properties by decreasing cell proliferation, damaging DNA, and inducing apoptosis, among other mechanisms. One of the most studied ROS is hydrogen peroxide (H_2O_2).

2. Hydrogen Peroxide

H_2O_2 is a protonated form of O_2^{2-} and is produced in biological systems by the dismutation of superoxide anion in a reaction carried out by the enzyme superoxide dismutase (SOD) in the following manner [9]:

$$\begin{aligned} M^{(n+1)+}\text{-SOD} + O_2^- &\longrightarrow M^{n+}\text{-SOD} + O_2 \\ M^{n+}\text{-SOD} + O_2^- + 2H^+ &\longrightarrow M^{(n+1)+}\text{-SOD} + H_2O_2, \end{aligned} \quad (1)$$

where M = Cu($n = 1$); Mn($n = 2$); Fe($n = 2$); Ni($n = 2$).

H_2O_2 is also a soluble lipid and strong oxidizing agent that has been suggested to diffuse throughout the cell membrane via some aquaporins like aquaporin-8, AtTIP1;1, and AtTIP1;2 [10, 11]. H_2O_2 is also a hypochlorous acid precursor [9, 12]. This ROS reacts in the presence of transition metals like cupper or iron and produces the hydroxyl radical, a powerful reactive and toxic compound.

One of the preferred targets for H_2O_2 is the DNA; it produces single- or double-stranded DNA breaks as well as DNA cross links, in addition to purine, pyrimidine, or deoxyribose modifications [13]. Changes in DNA are usually repaired by the cell, but when persistent DNA damage occurs, then replication errors, genomic instability, activation of oncogenes, and inactivation of tumor suppressor genes might emerge [14]. All of these processes have been associated with the development of a variety of cancers. However, increasing evidence shows that H_2O_2 has contrasting effects on cancer cell proliferation depending on its concentration; it generates several antiproliferative responses, induces apoptosis, and inhibits cancer cell migration and invasion.

3. Effects of H_2O_2 on Plasma Membrane and Calcium Mobilization

Ion channels play important roles in health and disease and Ca^{2+} signaling is an important second messenger that participates in many processes including proliferation and apoptosis. H_2O_2 increases intracellular Ca^{2+} concentration and decreases electrical resistance in human lung microvascular endothelial cells via activation of TRPV4 ion channels, through a mechanism that requires the Src tyrosine kinase Fyn [15]. In addition, exposure to H_2O_2 increases intracellular Ca^{2+} concentration in rat alveolar type II epithelial cells [16] and induces calcium release from the endoplasmic reticulum in endothelial cells [17]. Ma and collaborators observed that A549 lung cancer cells treated with H_2O_2 (500 μM) showed an intracellular Ca^{2+} elevation due to Ca^{2+} influx and Ca^{2+} mobilization from intracellular stores. They also describe that H_2O_2 increases polyethylenimine/oligonucleotide endocytosis by activating the calcium/calmodulin-dependent protein kinase II (CaMKII). This study suggests that H_2O_2 may be useful to improving aerosol oligonucleotide therapy in pulmonary diseases [18]. Zhang et al. also observed that H_2O_2 increases the cytoplasmic Ca^{2+} concentration in A549 cells [19]; this change in calcium concentration might be a critical regulator of apoptosis. Another plasma membrane effect of H_2O_2 is on adhesion molecules that are important for permeability and signaling transduction in lung epithelium [20]. When A549 cells were stimulated by H_2O_2 the levels of the adhesion molecules CD49f, CD49b, CD29, and CD44 were increased. The expression of these molecules is closely associated with the stress response [21]. The effect of H_2O_2 on the plasma membrane and intracellular calcium concentration may be already involved in triggering cell death (Figure 1).

4. H_2O_2 Induces Nuclear and Mitochondrial DNA Damage

Cells are constantly exposed to reactive oxygen species including those metabolically generated as products of aerobic respiration [32, 33] and those originated from environmental pollutants [34]. It has been observed that hydrogen peroxide concentrations above 100 μM are cytotoxic and genotoxic in A549 cells [35] causing DNA damage [36] and inducing the catalytic activities of DNA topoisomerase complexes [37–39]. Furthermore, the H_2O_2-induced damage could be also revealed by the oxidation of DNA bases, for instance, guanine adducts like 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) [40]. H_2O_2 DNA damage triggers a complex network of DNA damage response (DDR) pathways that may initiate DNA repair, arrest cell cycle progression, and trigger apoptosis. In A549 cells, H_2O_2 activates DDR through the Mre11 (MRN) complex of proteins (Mre11, Rad50, and Nbs1), which are essential for activation of telangiectasia mutated protein kinase (ATM), checkpoint kinase 2 (Chk2), and H2AX (γH2AX). After Chk2 activation, the cells become arrested at either the G2-M or G1-S transition [41]. Moreover, total p53 and $p21^{Cip1/Waf1}$ levels were increased after exposure of A549 cells to H_2O_2 [42]. These DNA damage response events induce the formation of DNA damage foci that probably will be activated by stalled replication forks, as well as by the induction of DNA double-strand breaks (DSBs) at the primary DNA lesion sites [43]. It has been described

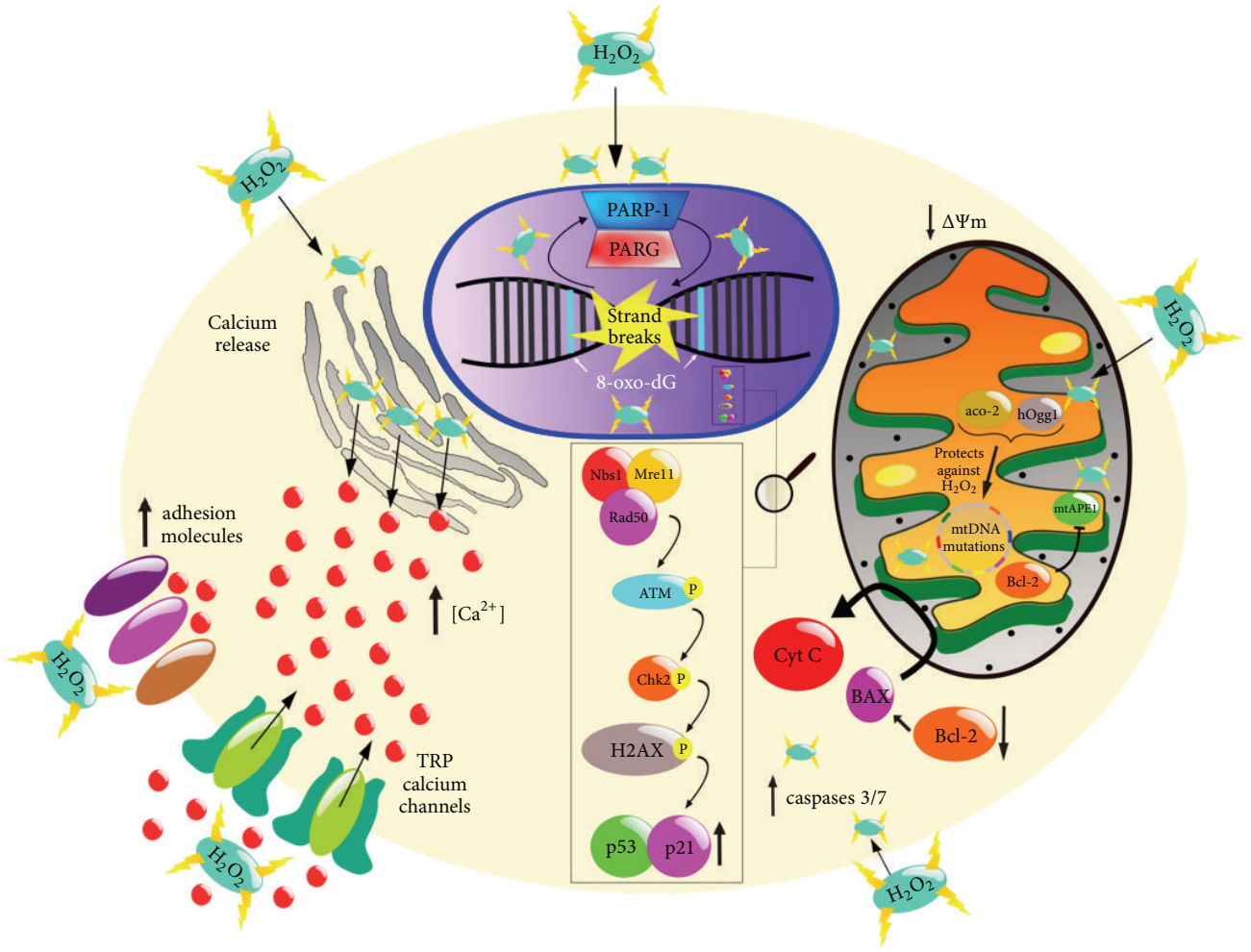

Figure 1: Effects of H_2O_2 that may lead to cell death. H_2O_2 affects several compartments and proteins potentially leading to cell death. Oxidation of DNA bases enriched with guanine adducts like oxo8dG base ring fragmentation, sugar modification, covalent cross linking of DNA and protein, and induction of DNA strand breaks may occur as a result of oxidative DNA damage induced by H_2O_2 [22]. Chk2 plays a major role in arresting the cell cycle progression in response to DNA damage [23]. Phosphorylation of Cdc25C and Cdc25A by Chk2 prevents cell cycle progression [24]. DDR involves the activation of the kinases ATM and Chk2 and their downstream effector p53 and its target $p21^{Cip1/Waf1}$ axis [25, 26]. Overexpression of Aco-2 reduced oxidant-induced mtDNA lesions, mitochondrial p53 translocation, and apoptosis. Bcl-2 family proteins control the relocalization and actions of cytochrome C, a relevant step of apoptotic cell death [27]. H_2O_2 also increases caspase-3/caspase-7 activity [28] and upregulates the cleaved-caspase-9 [27], modifying the $\Delta\Psi m$ [28]. Apoptosis is also related with increased Ca^{2+} concentration that may be increased by influx via TRP ion channels or released from intracellular stores [29–31].

that H_2O_2 activates poly(ADP-ribose) polymerase (PARP) enzymes when DNA strand breaks have been paired, with the activation of PARP-1 and poly(ADP-ribose) glycohydrolase (PARG), suggesting that this activation process is a survival mechanism. Three members of the 17-member PARP family (PARP-1 to PARP-3) have been shown to be activated by DNA damage. Activated PARP enzymes cleave NAD^+ into nicotinamide and ADP-ribose from which protein-bound (ADP-ribose)n polymers are synthesized; these polymers label the site of DNA damage enhancing DNA repair and consequently cell survival [44, 45]. Even though PARP activation has a central role in DNA single-strand break repair, its overactivation can cause cell death if excessive oxidative stress exists (in which DNA damage is severe and irreversible) [44]. On the other hand, repair of some DSBs can be error-prone resulting in deletion of base pairs and other defects that can result in translocations and chromosomal instability [46–49]. The association between DNA oxidation and DNA methylation in A549 cells exposed to H_2O_2 has been reported by Ke et al. Hydrogen peroxide induced the formation of 5-methylcytosine (5-mC), which is a cytosine variant produced by the transfer of a methyl group to the carbon located in the fifth position of cytosine. These authors showed that H_2O_2 induced decreased levels of DNA methylation in a dose-dependent manner, although significant changes in the level of DNA methylation required at least 10 days of exposure to the oxidant. This negative correlation suggests that DNA oxidation may take place before DNA methylation [40].

Not only nuclear DNA (nDNA) but also mitochondrial DNA (mtDNA) can be damaged by hydrogen peroxide.

The rate of mtDNA mutations may actually be more than two orders of magnitude higher than that of nDNA. Somatic mutations of mtDNA are potentially more harmful for cell physiology compared to somatic damage of nDNA; consequently, the DNA repair systems may play a more important role in the mitochondria than in the nuclei, especially in nondividing cells [50]. This could be explained because mtDNA is in close proximity to the electron transport chain and due to the lack of protective histones [51, 52]. Even though the mechanisms that modulate mtDNA damage are still unclear, Kim et al. suggest that human 8-oxoguanine DNA glycosylase (hOgg1) and aconitase-2 (aco-2) are important factors in limiting oxidant-induced mitochondrial DNA damage. Thus, H_2O_2 induces nuclear and mitochondrial DNA damage by several mechanisms [53]. Human APE1 is a major component of the base excision repair in both nDNA and mtDNA [54] in various types of cells, including lung cancer cells [7, 55]. It has been shown that Bcl-2 suppresses mtDNA repair through direct interaction with APE1 in mitochondria via its BH domains and inhibition of mtAPE1 endonuclease activity [56]. This led to increased frequency of mtDNA mutations following H_2O_2 or nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (a carcinogen in cigarette smoke) exposure in H1299 human lung cells [56]. Moreover, increased mitochondrial DNA (mtDNA) lesions in A549 cells have been reported after exposure to H_2O_2 in a dose-dependent manner; the effect also included a slight reduction in mtDNA copy number [53]. Figure 1 summarizes the effects of H_2O_2 on DNA damage.

5. Paradoxical Effects of H_2O_2 on Cancer Cell Proliferation and Migration

ROS have been proposed to have contrasting effects on cancer models. On one hand, ROS may promote cancer initiation; however, they can also inhibit metastasis of melanoma cells [57, 58]. Opposite or dual effects for H_2O_2 on cancer cell proliferation have also been described. For instance, H_2O_2 (50–200 μM) inhibits the proliferation of human breast cancer MCF-7 cells [59], but at 1–10 μM it increases the proliferation of hepatoma 7721 cells [60]. Interestingly, the proliferation of HT-29 colon cancer cells is enhanced at 10 μM whereas a higher concentration (1000 μM) leads to apoptosis [61]. H_2O_2 (50 μM) also produced cell cycle arrest in A549 lung cancer cells; this effect correlated with the downregulation of cyclins D1 and E [62]. Cell migration and invasion are very relevant in cancer progression and malignancy. Opposite effects of H_2O_2 on these phenomena have also been observed. The migration of H460 large lung cancer cells was inhibited by 100 μM H_2O_2; the superoxide anion and hydrogen peroxide downregulated Cav-1 expression and inhibited cell migration and invasion, whereas the hydroxyl radical upregulated Cav-1 expression and promoted cell migration and invasion. The downregulating effect of superoxide anion and hydrogen peroxide on Cav-1 was mediated through a transcription-independent mechanism that involved protein degradation via the ubiquitin-proteasome pathway [63]. In H1299, non-small lung cancer cells, 100 μM of H_2O_2 inhibited migration, upregulated Deleted in Liver Cancer 1 (DLC1) protein expression, and reduced the activity of RhoA [64]. Thus, H_2O_2 may be used as an inhibitor of cancer cell proliferation, migration, and invasion if used at particular concentrations and cancer cell types. The potential use of this ROS as an anticancer agent is also supported by its proapoptotic properties, as the following discussed.

6. Hydrogen Peroxide Leads to Cell Death/Apoptosis

DNA damage responses usually end up with the decrease of cell viability and activation of apoptosis pathways depending on the stimulus intensity. H_2O_2 induces cell death/apoptosis [65–67] and attenuates cell viability of A549 cells in a concentration- and time-dependent manner [68]. One of the first damage mechanisms induced by oxidative stress is carbonylation of lipids, proteins, and DNA as it has been observed in A549 lung cancer cells [69]. Moreover, H_2O_2 decreases intracellular ATP levels and stimulates caspase-3/caspase-7 activity [28] and upregulates the expression of cleaved-caspase-9 [27]. This nonradical ROS also affects the mitochondrial membrane potential, closely related to mitochondrial-mediated apoptosis [28]. Cui et al. showed that H_2O_2 downregulates the antiapoptotic protein Bcl-2, upregulates the proapoptotic protein BAX, and increases cytochrome C (Cyt C) release from the mitochondria (Figure 1) [27]. It is well known that translocation of BAX from the cytosol to the mitochondria plays a role in the release of mitochondrial proteins [70]. Prolonged dissipation of mitochondrial membrane potential ($\Delta\Psi$m) might result from mitochondrial DNA damage. Therefore, the upregulation of BAX and the loss of the $\Delta\Psi$m produced by H_2O_2 may be responsible for the effect of BAX in the mitochondrial release of Cyt C in A549 cells. Indeed, the apoptotic intrinsic pathway is activated by several mitochondrial proteins released into the cytosol, including Cyt C [71]. In addition to its effects on apoptosis, H_2O_2 (100 μM, 6–24 hours) induced necrosis in A549 cells [72]. Thus, H_2O_2 leads to cell death in different manners. Additionally, this ROS has effects on inflammation, as reviewed in the next section.

7. Inflammation, ROS, and H_2O_2 in Lung Cancer

Chronic inflammation has been proposed to play a central role in cancer development. Cancer-related inflammation is associated with the proliferation and survival of malignant cells, angiogenesis, tumor metastasis, and tumor response to chemotherapeutic drugs and hormones [73]. Thus, inflammation is a potential target for lung cancer prevention and treatment. Inflammatory cells release a variety of cytokines, chemokines, cytotoxic mediators including ROS, metalloproteinases (MMPs), and membrane-perforating agents, and soluble mediators of cell death, such as TNF-α (Tumor Necrosis Factor-α), interleukins (IL), and interferons (IFNs) [74]. The tumor stroma of NSCLC is characterized by active angiogenesis and abundant inflammatory infiltrate, which is mainly

composed of tumor-associated macrophages (TAM). It is also characterized by the presence of tumor infiltrating lymphocytes (TIL), including T, B, and natural killer (NK) cells, and tumor-associated neutrophils (TAN) [75, 76]. Several important molecules involved in the inflammatory response are regulated by or have been associated with ROS and H_2O_2.

7.1. TGFβ. Transforming growth factor-β (TGFβ) is an immunosuppressive cytokine [77] that has a pleiotropic role in tumor biology and is frequently overexpressed in many cancers, including NSCLC [78–80]. TGFβ affects cell growth, proliferation, differentiation, and apoptosis [81]. High expression of TGFβ is a poor survival predictor in NSCLC [79]. Treatment of human malignant mesothelioma cells (HMM) with H_2O_2 promoted the epithelial-mesenchymal transition, as indicated by increased expression levels of vimentin, SLUG, and TWIST1 and decreased E-cadherin. Expression of stemness genes such as OCT4, SOX2, and NANOG was also significantly increased in HMM cells treated with H_2O_2. These gene expression changes were mediated via activation of hypoxia inducible factor 1 alpha (HIF-1α) and TGF-β1 [82].

7.2. Interleukins. Interleukin-10 (IL-10) is a multifunctional cytokine with both immunosuppressive and antiangiogenic functions; thus, it has both tumor-promoting and tumor-inhibiting properties [83]. Increased serum and peritumoral IL-10 levels have been reported in several malignancies [84], including lung cancer [83], suggesting a role for IL-10 in the tumor escape from the immune response. High IL-10 expression and increased serum concentrations of IL-10 in NSCLC patients have been shown to correlate with reduced survival [83]. IL-10 serum levels are higher in patients with metastatic disease in contrast to patients with localized tumors [85]. IL-10 favors tumor malignancy by promoting T cell apoptosis and tumor cell survival [86]. In lung carcinomas, IL-10 inhibits tumor cell susceptibility to cytotoxic T-lymphocyte-mediated killing [87]. Transgenic mice overexpressing IL-10 developed larger tumors than control mice when injected with Lewis lung carcinoma cells, suggesting that the production of IL-10 prevents a full immune response against the tumor cells [88]. IL-6 is of particular interest because it is expressed in malignant epithelial cells, and their expression is associated with a poor prognosis in lung cancer patients [89]. This interleukin has been detected in primary squamous cell carcinomas, adenocarcinomas, and several tumor cell lines [90, 91]. In a study with lung cancer patients, increased serum levels of IL-6 were found in 39% of the patients, whereas it was not detected in the serum of healthy controls or in patients with benign lung diseases [90, 92]. Bihl and coworkers demonstrated that IL-6 may be required for the proper control of cell proliferation in a subset of NSCLC cell lines. Two cell subgroups were reported in this study: NSCLC IL-6-dependent and IL-6-independent cells; this finding may have interesting clinical implications [93]. Paradoxically, antitumor effects of IL-6 have been demonstrated *in vitro* and *in vivo*, as well as in human biopsies from NSCLC and breast cancer [90]. TNF-α induced IL-8 gene expression in H441 lung epithelial cells by activating the IL-8 promoter via recruitment of NF-κB to a TNF-α response element [94]. Similar results were obtained with lung adenocarcinoma GLC-82 cells treated with H_2O_2 (0.5 mM) [95]. In addition, Hsu et al. described that A549 lung cancer cells treated with H_2O_2 showed reduced I-$\kappa\beta$ expression with a concomitant increase in NF-κB and IL-8 expression [21].

7.3. NF-κB. NF-κB is a positive mediator of cell growth and proliferation as well as a critical signaling molecule in H_2O_2-induced inflammation. NF-κB increases the expression of several components involved in cell cycle progression including cyclins D and E. However, the contributions of NF-κB to lung cancer development are complex, and the underlying mechanisms are not fully understood [96]. Tumor biopsies from lung cancer patients showed high levels of NF-κB activation in both SCLC and NSCLC and were significantly associated with TNM (tumor size, node status, and metastasis) stages and poor prognosis [96]. Interestingly, inhibiting NF-κB with either siRNA, IKK inhibitors, or IKK suppressors inhibited lung cancer cell survival and proliferation [96, 97]. H_2O_2 activates cytosolic phosphorylation of NF-κB p65 and ERK1/2 and induces nuclear translocation of pNF-κB p65 producing inflammatory damage in A549 lung cancer cells. The genes involved in this response of the NF-κB and MAPK signaling pathways included IL-1β, IL-6, IL-8, TNF-α, MCP-1, IP-10, and MIP [98].

7.4. MMPs. MMPs are a family of proteolytic enzymes that are capable of degrading various components of the extracellular matrix [99]. They are involved in all stages of cancer progression, not only in the process of tumor invasion and metastasis [100], but also in the proliferation, adhesion, migration, differentiation, angiogenesis, senescence, autophagy, apoptosis, and evasion of the immune system [101, 102]. Several studies have reported that plasma and/or serum levels of MMP-9 and TIMP-1 are elevated in stage III/IV lung cancer patients, when compared with patients with nonmalignant lung diseases [103, 104]. Retrospective studies of NSCLC tissue found that MMP-7 expression was higher in squamous cell carcinomas than in adenocarcinomas and correlated with significantly lower overall survival in patients [105]. MMP-9 is not produced by resident cells in the normal lung, but bronchial epithelial cells, alveolar type II cells, fibroblasts, smooth muscle cells, and endothelial cells produce MMP-9 in response to diverse stimuli [106]. Leukocytes in the lung can also be a source of MMP-9. Macrophages, eosinophils, mast cells, lymphocytes, NK cells, and dendritic cells all are able to produce MMP-9 [106]. Lung cancer cells, both primary and metastatic, can express MMP-9 constitutively, which may correlate with metastatic potential [106–108].

The transcription factor Ets-1 was found to be associated with the progression of several human cancers including NSCL [109]. Ets-1 may upregulate MMP-9 expression triggered by TGF-beta1 and TPA via MAPK signaling [110]. H_2O_2 upregulates Ets-1 via an antioxidant response element in the promoter, suggesting its potential role in ROS-triggered tumor progression [111]. Interestingly, H_2O_2 induced MMP-2 and MMP-9 expression in the lung adenocarcinoma cell line GLC-82, as well as of several components activated by the innate immune response including MyD88, TRAF2, TRAF6,

and TRADD [95]. The association of ROS with inflammation might be used to suggest combined treatments of H_2O_2 with anti-inflammatory drugs in cancer therapy.

8. Potential H_2O_2-Based Therapeutic Strategies and Implications

Herein we described that hydrogen peroxide has several effects on lung cancer cells including DNA damage, cell cycle arrest, apoptosis, migration, and inflammation. Because many of these mechanisms end up with cell death, cautious delivery of H_2O_2 may be used as a potential therapeutic tool to treat some disorders including lung cancer. Actually, opposite effects of H_2O_2 may be also used in favor of some conditions. For instance, H_2O_2 (30 μM) induced the migration of A549 cells, showing that the exposure to low concentrations of hydrogen peroxide may benefit tissue repair during acute lung injury [112]. Furthermore, H_2O_2 has been used to enhance the adhesion of hematopoietic stem/progenitor cells when systemically administered in inflammatory bowel disorders [113].

A few years ago, a hydrogen peroxide-generating system emerged as an interesting anticancer alternative strategy to selectively kill cancer cells. As cancer cells generate high concentrations of ROS and are under increased intrinsic oxidative stress, they might be more vulnerable to further oxidative insults produced by ROS-generating agents [114]. In malignant cells, prooxidant changes induce a redox shift that turns the cancer cell proliferative machinery on, leading to functional impairment, cell cycle arrest, and finally cell death. Even if the direct administration of H_2O_2 to cancer patients is not an accepted therapeutic strategy, there is now convincing evidence that H_2O_2-generating systems might be an efficient way of killing cancer cells [115]. For instance, H_2O_2 can selectively induce apoptosis in cancer cells and mediate, at least in part, the activity of several anticancer drugs including paclitaxel, doxorubicin, cisplatin, casiopeínas, and arsenic trioxide since these drugs generate ROS as a potential mode of action, increasing the rate of cancer cell death [116]. Actually, H_2O_2 seems to play an important role in oxidative stress-induced cancer cell death [115, 117]. H_2O_2 produced in the mitochondria is able to induce cell cycle arrest and senescence, a combination that might suppress tumor growth when sublethal concentrations of ROS are generated in response to therapy [118]. Not only synthetic products but also natural compounds have been described as promising candidates to potentially increase ROS levels and attack a wide variety of cancer cells. For instance, the codrug Bet-CA (a chemical combination of dichloroacetate and betulinic acid) increases ROS production and significantly alters mitochondrial membrane potential gradient ($\Delta\Psi$m), followed by the release of Cyt C which prompts cells to undergo mitochondria mediated apoptosis [119].

Recently, cold atmospheric or nonthermal plasma has been suggested as an alternative therapy for different types of cancers with promising results obtained *in vitro* [120, 121] as well as *in vivo* [122, 123]. Nonthermal plasma can be produced by ionizing neutral gas molecules/atoms, which leads to a highly reactive gas at room temperature. This gas contains excited molecules and reactive species, among its most important constituents [123]. The therapeutic effects of nonthermal plasma result from the generation of ROS, which lead to $\Delta\Psi$m, mitochondrial ROS accumulation, changes in the cell cycle, expression of DNA damage markers like γH2AX, and finally induction of apoptosis [120, 121, 123]. Nonthermal plasma decreases the intracellular ATP concentration and the viability of A549 cells. It also increases the number of apoptotic cells due to caspase activation. In addition, plasma alters the mitochondrial membrane potential, regulates the mRNA levels of BAX, BAX1, H2AX, and Bcl-2, and modifies phosphorylated ERK1/2/MAPK protein levels [28].

Panieri et al. demonstrated that NSCLC cells resistant to conventional anticancer treatment can be sensitized in the presence of either high levels of H_2O_2 (48 μM) resulting in DNA damage and irreversible ATP depletion (caspase-independent) or lower H_2O_2 concentrations (6.5 μM) which induces inhibition of glycolysis and abrogation of ATP restoring mechanisms. Thus, cancers not responding to conventional therapies may be evaluated for their response to different H_2O_2 concentrations. Despite the fact that H_2O_2 may activate the inflammatory response potentially leading to cancer, the combined use of H_2O_2 with anti-inflammatory drugs may preserve the anticancer effect of this ROS and overwhelm the potential inflammatory response improving the anticancer treatment.

Recently, several drugs indicated for other diseases have been shown to have antiproliferative properties and have been suggested as an alternative therapy for different malignancies including lung cancer [124]. Thus, the novel combination of H_2O_2 with such repositioned drugs represents a new research area in cancer therapy.

9. Conclusions

Because of the multimechanistic and multitarget anticancer properties of H_2O_2, this molecule is a very interesting potential therapeutic tool to fight cancer (Figure 2). The proper and cautious use of H_2O_2 in combination with commonly used chemotherapeutic drugs may have synergistic effects increasing lung cancer cell death. Particularly, novel therapeutic approaches combining H_2O_2 with repositioned drugs may help to decrease the mortality from this malignancy.

Abbreviations

aco-2:	Aconitase-2
APE1/ref-1:	Apurinic/apyrimidinic endonuclease 1/ref-1
APE1:	Purinic/apyrimidinic (AP) endonuclease 1
ATM:	Activation of telangiectasia mutated protein kinase
ATP:	Adenosine triphosphate
Bcl-2:	B cell lymphoma 2
CaMKII:	Calcium/calmodulin-dependent protein kinase II
Cav-1:	Caveolin-1
CD49f:	Cluster of differentiation
CDKs:	Cyclin dependent kinases
Chk2:	Checkpoint kinase 2

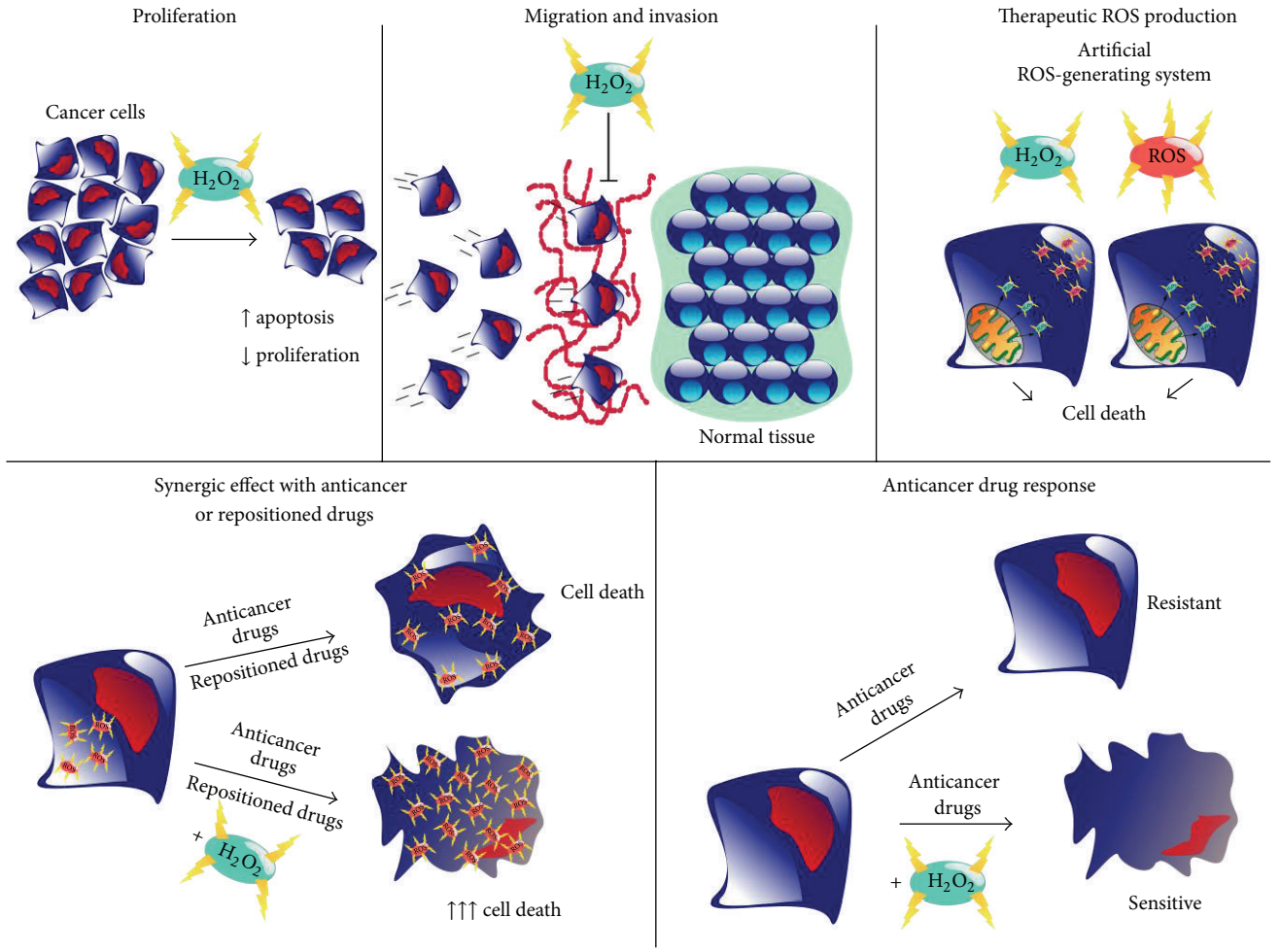

FIGURE 2: Potential therapeutic use of H_2O_2 to fight lung cancer. H_2O_2 can decrease the proliferation and increase the apoptosis of lung cancer cells. In addition, metastasis may be prevented because of the inhibitory effects of H_2O_2 in cell migration and invasion. Artificial ROS-H_2O_2 production directed to cancer cells in an excessive manner may lead also to cell death. H_2O_2 may also increase the cytotoxicity of anticancer drugs and revert drug resistance, as well as potentiating the effect of repositioned drugs with anticancer effects.

Cyt C: Cytochrome C
DDR: DNA damage response
DLC1: Deleted in Liver Cancer 1
DSBs: DNA double-strand breaks
ERK1/2: Extracellular-signal-regulated kinases
Fyn: Nonreceptor tyrosine kinase
H2AX: Histone family, member X
H_2O_2: Hydrogen peroxide
HIF-1α: Hypoxia inducible factor 1 alpha
HMM: Human malignant mesothelioma cells
hOgg1: Human 8-oxoguanine DNA glycosylase
IFNs: Interferons
IKK: IkB kinase
IL: Interleukins
IP-10: Interferon gamma-induced protein 10
MCP-1: Monocyte chemotactic protein 1
MIP: Macrophage Inflammatory Proteins
MMPs: Metalloproteinases
MRN: Complex of Mre11, Rad50, and Nbs1 proteins
mtDNA: Mitochondrial DNA
nDNA: Nuclear DNA
Nbs1: Nibrin
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NK: Natural killer
NSCLC: Non-small-cell lung cancer
OCT4: Octamer-binding transcription factor 4
PARG: Poly(ADP-ribose) glycohydrolase
PARP: Poly(ADP-ribose) polymerase
Rad50: Double-strand break repair protein
ROS: Reactive oxygen species
SCLC: Small-cell lung cancer
siRNA: Small interfering RN
SOD: Superoxide dismutase
SOX2: Sex determining region Y-box 2
Src: Nonreceptor tyrosine kinase
TAM: Tumor-associated macrophages
TAN: Tumor-associated neutrophils
TGFβ: Transforming growth factor-β
TIL: Tumor infiltrating lymphocytes

TIMP-1: Tissue metallopeptidase inhibitor
TNF-α: Tumor Necrosis Factor-α
TRPV4: Transient receptor potential cation channel subfamily V member 4
$\Delta\Psi m$: Mitochondrial membrane potential.

Disclosure

Gabriela Vilema-Enríquez is a recipient of a doctoral scholarship from the Ecuadorian government through the Secretariat for Higher Education, Science, Technology and Innovation (SENESCYT).

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Gabriela Vilema-Enríquez and Aurora Arroyo contributed equally to this work.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4650489, 10 pages
http://dx.doi.org/10.1155/2016/4650489

Research Article

The Analgesic Effect of the Mitochondria-Targeted Antioxidant SkQ1 in Pancreatic Inflammation

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Received 25 January 2016; Accepted 7 April 2016

Academic Editor: Angel Catalá

Background. Chronic pancreatitis is one of the main risk factors for pancreatic cancer. In acute and chronic pancreatitis, oxidative stress is thought to play a key role. In this respect, the recently described mitochondria-targeted antioxidant SkQ1 effectively scavenges reactive oxygen species at nanomolar concentrations. Therefore, we aimed to characterize the influence of SkQ1 on tissue injury and pain in acute and chronic pancreatitis. *Methods*. Both acute and chronic pancreatitis were induced in C57BL/6 mice by intraperitoneal cerulein injections and treatment with SkQ1 was carried out by peroral applications. Hyperalgesia was assessed by behavioral observation and measurement of abdominal mechanical sensitivity. Blood serum and pancreatic tissue were harvested for analysis of lipase and histology. *Results*. SkQ1 did not influence pain, serological, or histological parameters of tissue injury in acute pancreatitis. In chronic pancreatitis, a highly significant reduction of pain-related behavior ($p < 0.0001$) was evident, but histological grading revealed increased tissue injury in SkQ1-treated animals ($p = 0.03$). *Conclusion*. After SkQ1 treatment, tissue injury is not ameliorated in acute pancreatitis and increased in chronic pancreatitis. However, we show an analgesic effect in chronic pancreatitis. Further studies will need to elucidate the risks and benefits of mitochondria-targeted antioxidants as an analgesic.

1. Introduction

Acute and chronic pancreatitis rank within the most common causes for hospital admission among gastrointestinal disorders and represent a significant healthcare burden [1]. Furthermore, chronic pancreatitis represents a key risk factor predisposing to pancreatic cancer [2]. Despite intensive efforts, treatment is limited to supportive measures and none of the therapeutic approaches evaluated up until now have been shown to ameliorate the course of both acute and chronic pancreatitis. Oxidative stress has been proposed as a key pathophysiological factor [3, 4]. A common model of oxidative stress in pancreatitis suggests that oxidative stress is the result of cytochrome P450 induction, excess exposure to bioactivated metabolites, and a deficiency of reduced glutathione [3]. While evidence from animal studies suggests that antioxidant therapy could reduce inflammatory processes in pancreatitis [4–6], clinical studies were not able to demonstrate a relevant therapeutic effect [7, 8].

In chronic pancreatitis, especially, pain is a major clinical problem that affects up to 90% of patients and severely impacts quality of life [9, 10]. Furthermore, recurrent episodes of pancreatitis and pain are a relevant clinical problem in patients with pancreatic cancer [2]. Historically, pain in chronic pancreatitis has been thought to be caused by increased pancreatic pressure and mechanical strictures [11]. Current research on pain in chronic pancreatitis majorly focuses on pancreatic neuropathy and pancreatic neuritis

[12]. In accordance with the hypothesis that oxidative stress is critically linked to the pathogenesis of chronic pancreatitis and its symptoms, numerous studies have attempted to demonstrate an analgesic effect of antioxidants in chronic pancreatitis [13]. In this respect, a recent Cochrane analysis demonstrated that antioxidants only slightly reduce pain in chronic pancreatitis and their clinical value remains unclear [13].

However, the effect of antioxidants in pancreatitis has only been evidenced by the use of classical, non-mitochondria-targeted antioxidants. Mitochondria-targeted antioxidants have recently been shown to exert cytoprotective effects [14–20] in numerous studies and are thought to be effective at nanomolar concentrations [21]. Within the cytosol, mitochondria are the only anionic organelles and are specifically targeted by the cationic group of antioxidants of this family [22]. In this regard, two antioxidants, SkQ1 [23] and MitoQ [24], have been the focus of research in the field of mitochondria-targeted antioxidants. Of these, SkQ1 has been shown to be effective at lower concentrations [23, 25] and has been demonstrated to ameliorate trauma-induced neurological deficit [16], protect erythrocytes from oxidative hemolysis [17], reduce TNF-α induced endothelial damage [18], modulate angiogenesis [26], and decrease ischemia-reperfusion injury in liver transplantation [20]. On a molecular level, the antioxidative effect of SkQ1 relies on plastoquinone, an electron carrier in photosynthesis [23].

As a highly effective scavenger of reactive oxygen species (ROS), we hypothesize that the mitochondria-targeted antioxidant SkQ1 [23] would reduce inflammatory cell damage in mouse models of acute and chronic pancreatitis. As such, serological and histological parameters were evaluated. Furthermore, we aimed to investigate whether SkQ1 would affect pancreatitis-associated pain as measured by behavioral pain testing.

2. Material and Methods

2.1. Animals. Female C57BL/6 mice obtained from Charles River (Sulzfeld, Germany) were kept in accordance with regulations of the Federal Republic of Germany with a 12-hour light-dark cycle and food and water ad libitum. All animal procedures were performed according to local ethical guidelines and approved by the district government of Upper Bavaria (55.2-1-54-2532-165-2014). Cerulein (50 μg/kg/injection in saline; Sigma, Sigma Life Science, St. Louis, USA) or saline (control) was administered intraperitoneally 8 times at hourly intervals for the induction of acute pancreatitis. For experiments on chronic pancreatitis, mice received cerulein (50 μg/kg/injection in saline) or saline (control) at five hourly injections of cerulein three times a week over a period of eight weeks. Antioxidative treatment with SkQ1 (10-(6′-plastoquinonyl)decyltriphenylphosphonium) [27] was administered perorally with the drinking water at a dose of 5 nmol/kg body weight per day (on average, a mouse drank about 5 mL of water per day).

For experiments on both acute and chronic pancreatitis, mice were divided in three groups. Group A (acute pancreatitis (AP) $n = 8$; chronic pancreatitis (CP) $n = 12$) was treated with 5 nmol/kg SkQ1, group B (AP $n = 8$; CP $n = 12$) was the untreated control, and group C (AP $n = 8$; CP $n = 7$) was the sham group, which was injected intraperitoneally with 0.9% NaCl instead of cerulein and was therefore the negative control group without pancreatitis.

For experiments on acute pancreatitis, mice were pretreated with SkQ1 for 8 weeks prior to induction of pancreatitis. Mice designated for experiments on chronic pancreatitis received SkQ1 at the same concentration for 8 weeks in parallel with induction of pancreatitis.

2.2. Open-Field Test/Vertical Activity. Behavioral measurement of nonevoked pain-related behavior was conducted as described before [28]. Originally, the open-field test was developed to measure emotionality by qualitative and quantitative measures of locomotion. Here, we focus on the vertical activity (rearing) as the main outcome parameter. Rearing is an explorative behavior that has been shown to mirror anxiety which closely correlates with pain sensation in rodents. Less vertical activity is, therefore, interpreted as more anxiety and pain. Briefly, mice were allowed to move freely in a square box of 70 × 70 × 60 cm. After an accommodation period of 10 minutes, behavior was recorded via a video camera over a time period of five minutes and vertical activity was determined by counting the times the mice reared up. Between the observations, the box was thoroughly cleaned with ethanol to eliminate olfactory cues from previous subjects. Vertical activity was assessed once before the first administration of cerulein and once after administration of cerulein was completed.

2.3. Von Frey's Test. Mechanical sensitivity and evoked pain-related behavior was measured using Von Frey's hairs. The mice were allowed to move freely in a transparent box of 10 × 10 × 10 cm placed on a metal-lattice. The boxes were cleaned with ethanol to eliminate odor from other subjects. Following an accommodation period of 10 minutes, the abdomen was stimulated with a series of Von Frey's hairs of 0.25, 0.5, 1.0, and 2.0 mN with 10 applications each. Reactions to stimulation with Von Frey's hairs were graded and a pain score was build. A response was defined as an arousal reaction and allocated with one point. Any type of defense or flight reaction was allocated with two points. A cumulative score representing the sum of pain scores of all filaments was used to assess mechanical hypersensitivity.

2.4. Serum Lipase. Blood samples were obtained via ventricular heart puncture before sacrifice. Serum lipase levels were measured in the central clinical laboratories (Zentrallabor, Hospital of the University of Munich, Germany) according to locally defined guidelines.

2.5. Histology. Hematoxylin and eosin staining of paraffin sections was performed according to standard protocols. Subsequently, slides were analyzed by an experienced pathologist (JN) and assessed according to Spormann's score [29, 30]. Tissue edema and neutrophil infiltration were both graded on a scale from zero to three, and parenchymal necrosis and fat necrosis were separately graded on a scale from zero to

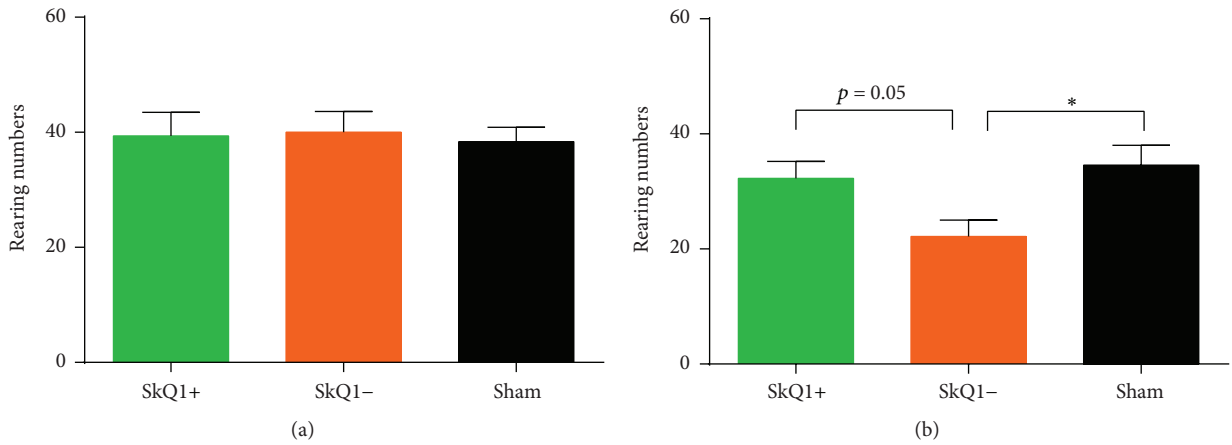

Figure 1: Vertical activity in acute (a) and chronic (b) pancreatitis: displayed are the rearing numbers that were counted during the observation time. While there were no differences between the groups in acute pancreatitis (a), treated mice (SkQ1+) with chronic pancreatitis showed significantly more activity than the untreated (SkQ1−) and saline controls (sham), suggesting less pain in the treated mice (b). *Statistically significant results ($p < 0.05$).

seven. The total score consisted of the sum of scores for tissue edema, neutrophil infiltration, parenchymal necrosis, and fat necrosis. Additionally, paraffin sections were stained with aniline blue to assess the degree of fibrosis.

2.6. Statistical Analysis. Statistical analysis was done using one-way ANOVA and Tukey's test for multiple comparisons. The α-level was set at 0.05. Results are displayed as means and their respective standard error of the mean (SEM). Data analysis was carried out using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com/).

3. Results

3.1. Acute Pancreatitis

3.1.1. Nonevoked Pain-Related Behavior. The number of observed rear ups was not statistically significant between both groups A (39.38 ± 4.09) and B (40.00 ± 3.65) or the sham group. Additionally, no statistically significant differences were evident between groups A and B ($p = 0.99$). Figure 1(a) compares the vertical activity of all groups after induction of acute pancreatitis.

3.1.2. Evoked Pain-Related Behavior. Baseline experiments did not reveal statistically significant differences between groups A, B, and C (Figure 2(a)). The cumulative pain scores of groups A and B were significantly different from the sham group (Figures 2(b) and 2(c)). However, we detected a tendency towards SkQ1 attenuating evoked pain in mice affected by pancreatitis. Pain scores for group A were 9.38 ± 0.20 and for group B 10.26 ± 0.27 ($p = 0.08$).

3.1.3. Serum Lipase. Serum lipase levels of groups A (1887 ± 247 U/L) and B (1856 ± 433 U/L) significantly differed from the sham group (Figure 3(a)). No statistically significant differences were seen in the comparison of groups A and B ($p = 0.99$). Figure 3(a) provides a graphical display of serum lipase levels in acute pancreatitis.

3.1.4. Histological Grading. As measured by Spormann's score (Figures 4(a) and 7(a)–7(c)), groups A and B displayed statistically significant differences from the sham group. The difference between groups A (6.87 ± 1.25) and B (8.00 ± 1.48) was not statistically significant ($p = 0.77$; Figures 4(a) and 7(a)–7(c)). Furthermore, a subanalysis of the parameters edema, neutrophil infiltration, parenchymal necrosis, and fat necrosis also did not show statistically significant differences between groups A and B (Figures 5(a)–5(d)).

3.2. Chronic Pancreatitis

3.2.1. Nonevoked Pain. No statistically significant differences were evident at baseline measurements between groups A and B and the sham group. After week four, groups A and B showed statistically significant differences from the sham group, while a statistically significant difference between groups A (28.42 ± 2.82) and B (24.00 ± 3.38) was not seen ($p = 0.62$). Measurement of vertical activity at the end of the experiment at week eight (Figure 1(b)) revealed significantly increased vertical activity in group A with rearing of 32.25 ± 2.98 versus 22.17 ± 2.88 in group B ($p = 0.05$). While the mean of group B significantly differed from the sham group, the mean of group A did not (Figure 1(b)).

3.2.2. Evoked Pain. At baseline, no statistically significant differences were evident in the pain scores between both groups A and B and the sham group (Figure 8(a)). Moreover, mean pain scores between groups A (3.60 ± 0.35) and B (2.79 ± 0.37) also did not display statistically significant differences ($p = 0.24$; Figure 8(a)). After week four, still no difference between groups A (6.05 ± 0.50) and B (6.92 ± 0.55) was detected ($p = 0.44$; Figure 8(b)). But both groups A and B differed significantly from the sham group (Figure 8(b)). At week eight (Figures 8(c) and 8(d)), the cumulative pain score

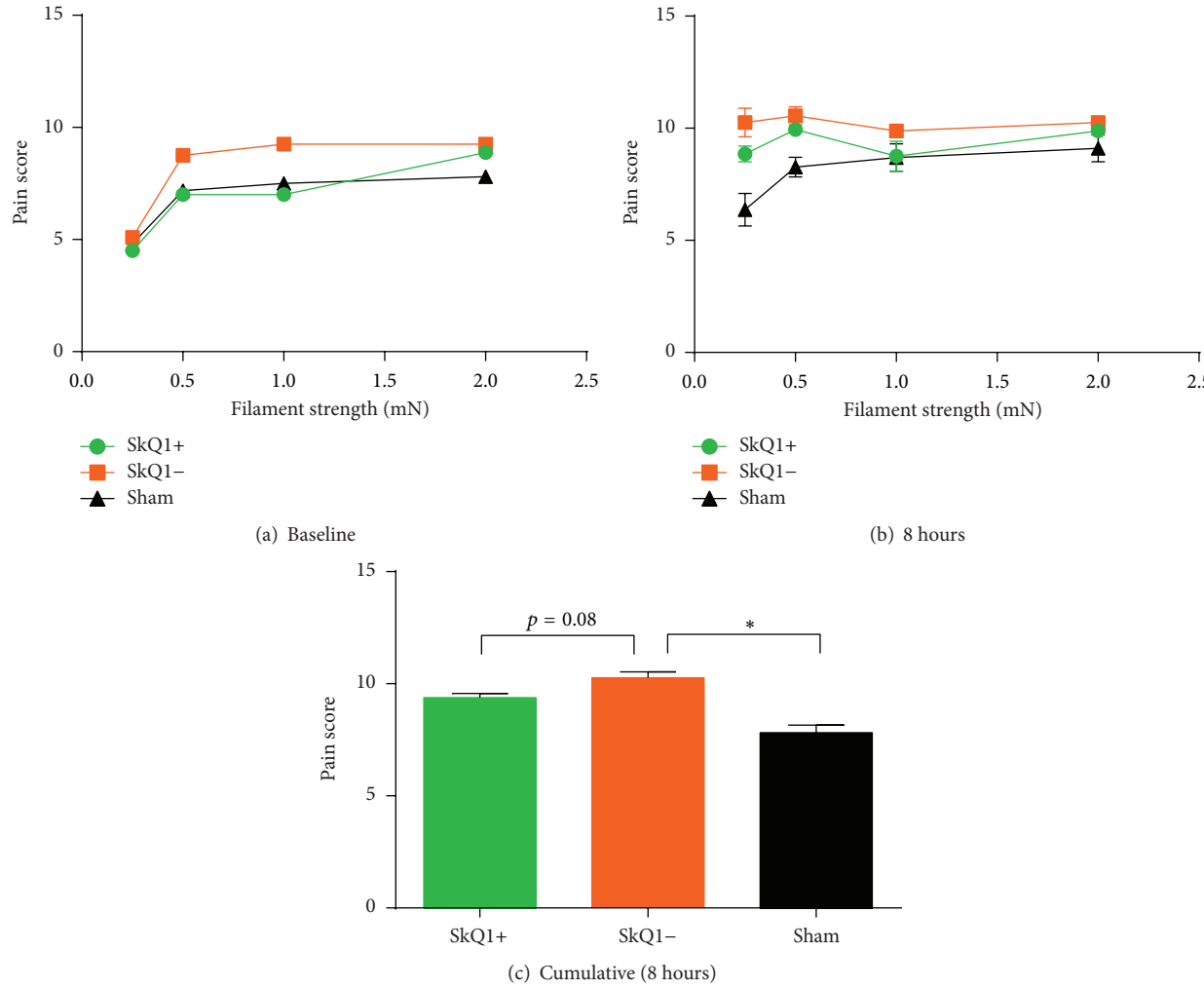

Figure 2: Evoked pain-related behavior measured by Von Frey's hairs in acute pancreatitis: both baseline and final measurements did not show any significant differences between the groups. *Statistically significant results ($p < 0.05$).

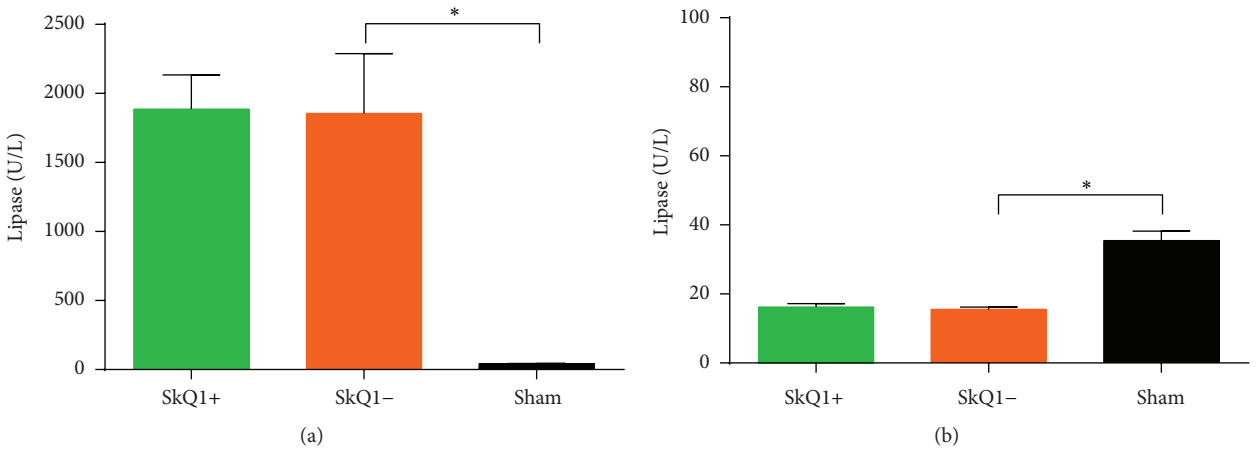

Figure 3: Serum lipase levels in acute (a) and chronic pancreatitis (b): while mice with acute pancreatitis showed significantly higher levels of serum lipase than the saline controls, no differences were observed with or without treatment (a). In chronic pancreatitis, mice showed lower serum lipase levels than the saline controls, reflecting advanced disease. As in acute pancreatitis, no differences were observed in treated versus untreated mice (b). *Statistically significant results ($p < 0.05$).

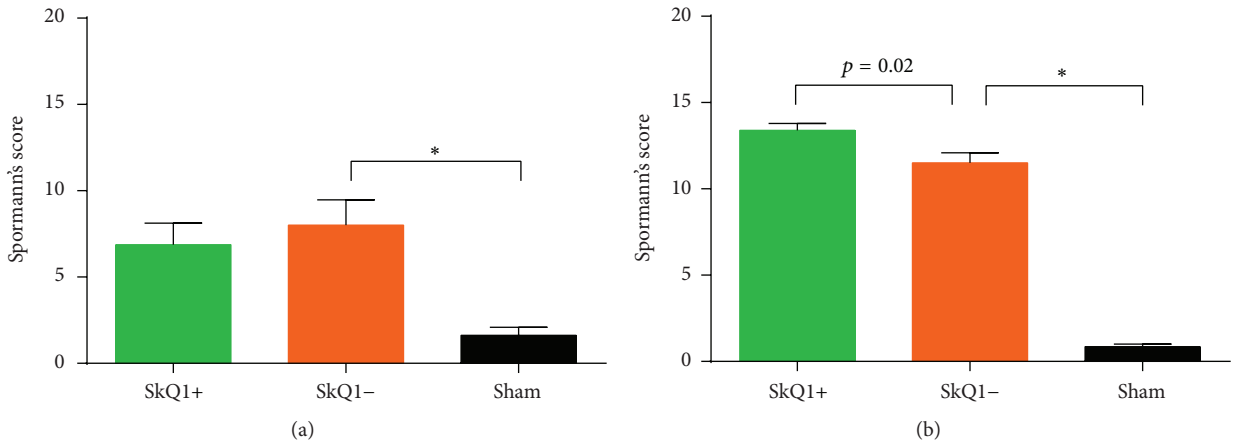

Figure 4: Histological severity of acute (a) and chronic pancreatitis (b) measured by Spormann's score: in acute pancreatitis, no differences were observed in treated versus untreated mice (a). In chronic pancreatitis mice treated with SkQ1 showed a slightly higher tissue damage when compared to the untreated mice (SkQ1−) (b). *Statistically significant results ($p < 0.05$).

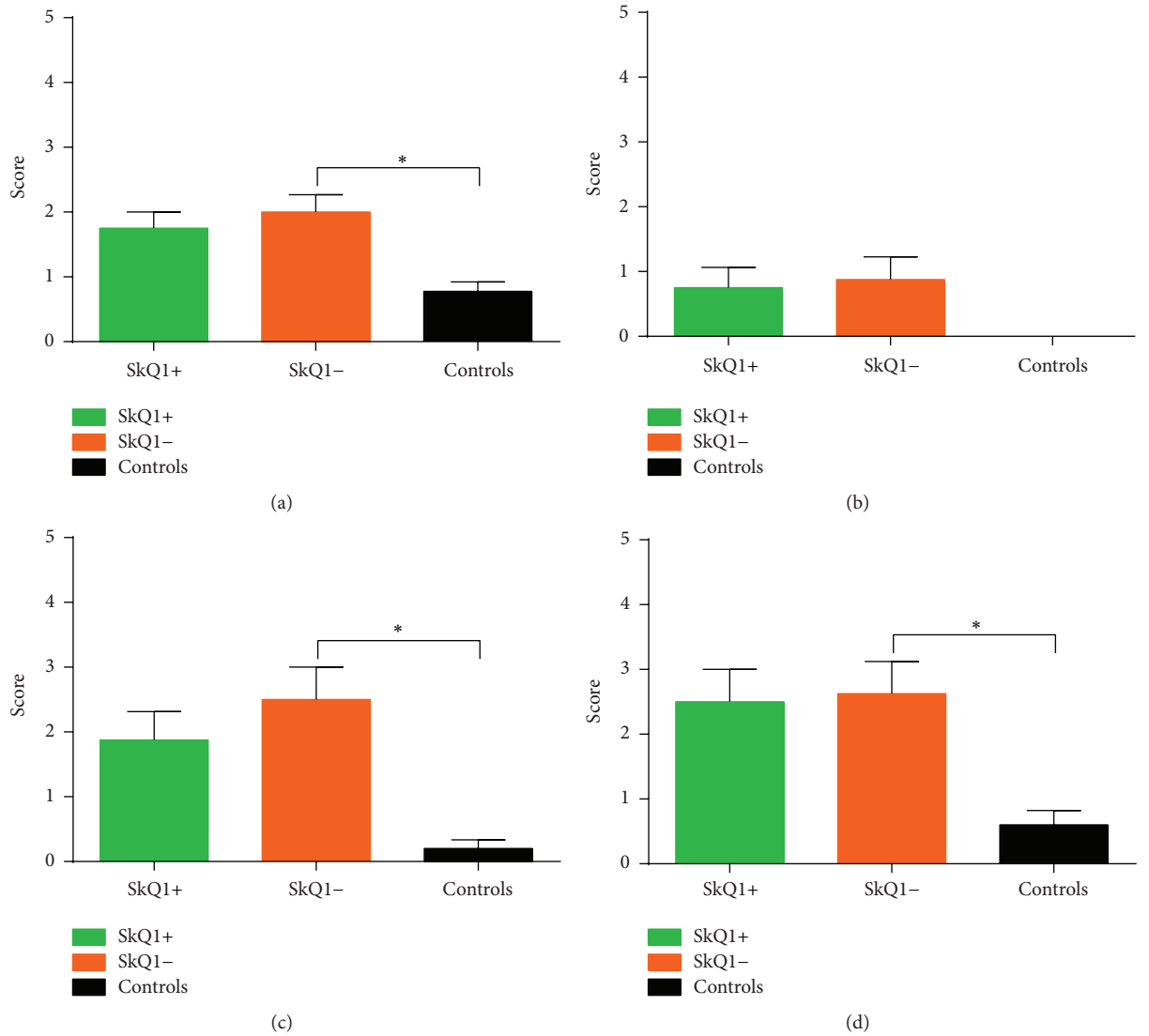

Figure 5: Subparameters of histological severity in acute pancreatitis as measured by Spormann's score: neither edema (a) nor neutrophil infiltration (b) nor parenchymal necrosis (c) nor fat necrosis (d) showed statistically significant differences between the SkQ1+ and SkQ1− groups. *Statistically significant results ($p < 0.05$).

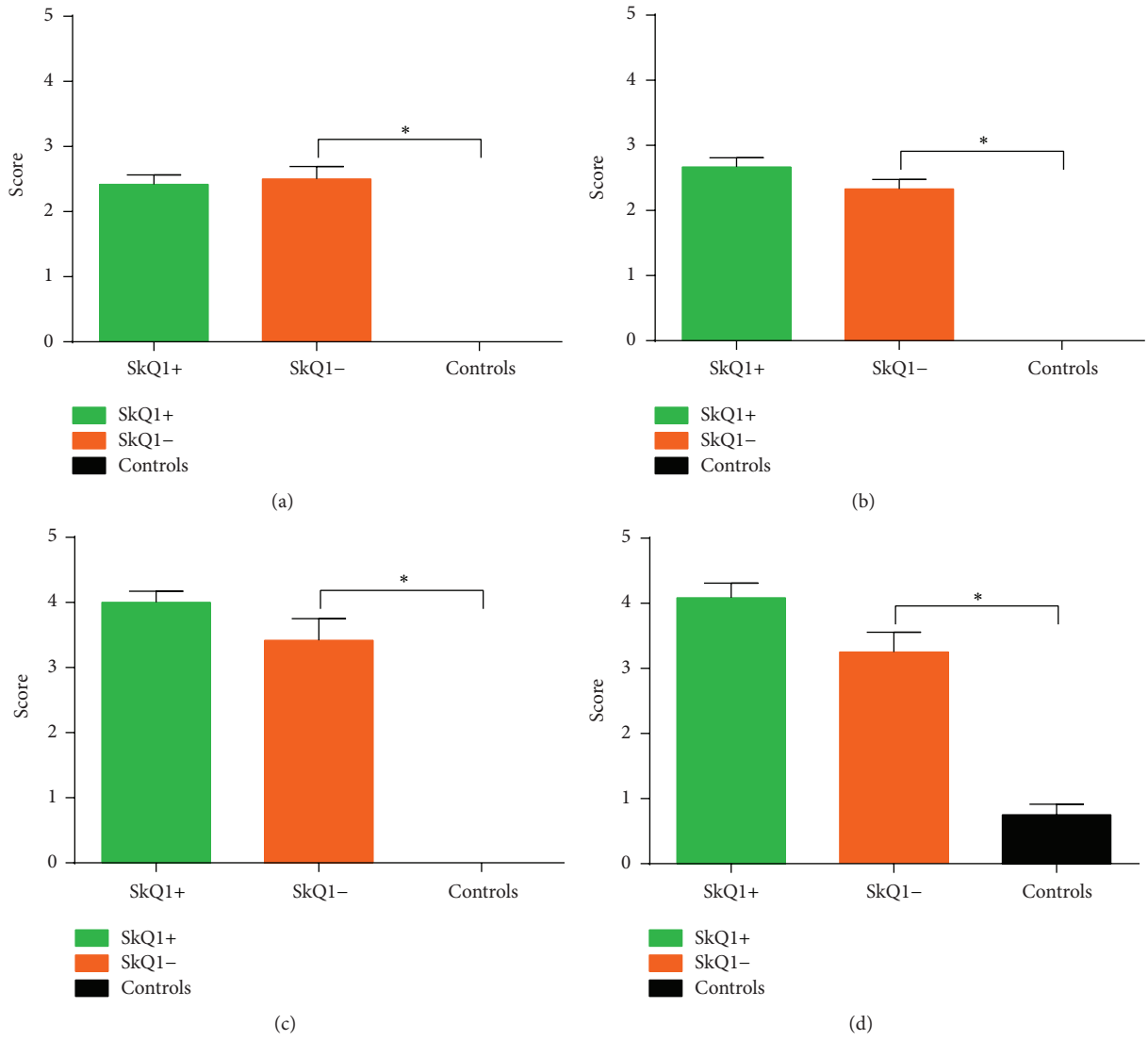

Figure 6: Subparameters of histological severity in chronic pancreatitis as measured by Spormann's score: again, none of the subparameters (edema (a), neutrophil infiltration (b), parenchymal necrosis (c), and fat necrosis (d)) showed statistically significant differences between the SkQ1+ and SkQ1− groups. *Statistically significant results ($p < 0.05$).

of group A (6.23 ± 0.39) significantly differed ($p < 0.0001$) from the pain score of group B (9.83 ± 0.70). When the sham group and group A were compared, no statistically significant difference was evident ($p = 0.72$; Figure 8(d)). The pain score of group B was statistically different from the sham group ($p < 0.0001$).

3.2.3. Serum Lipase. Measurement of serum lipase did not reveal statistically significant differences between groups A (16.20 ± 1.00 U/L) and B (15.50 ± 0.74 U/L; $p = 0.92$; Figure 3(b)). Both groups A and B displayed statistically significant differences from the sham group. Figure 3(b) displays serum lipase levels of groups A–C.

3.2.4. Histological Grading. Analysis of Spormann's score (Figures 4(b) and 7(d)–7(f)) revealed statistically significant differences between both groups A and B and the sham group. Spormann's score of group A was significantly higher than Spormann's score of group B (13.40 ± 0.40 versus 11.50 ± 0.58, resp.; $p = 0.02$; Figures 4(b) and 7(d)–7(f)). No statistically significant differences were detected with respect to the subparameters edema, neutrophil infiltration, parenchymal necrosis, and fat necrosis (Figures 6(a)–6(d)). Anilin staining for fibrosis confirmed the histological findings (data not shown).

4. Discussion

Chronic pancreatitis has been identified as a major risk factor for pancreatic cancer [2] and despite an abundance of studies and growing understanding of the pathophysiology of acute and chronic pancreatitis, there are still no specific therapeutic options [8, 31]. Experimental and clinical studies suggest a correlation of the oxidative burden and disease severity in

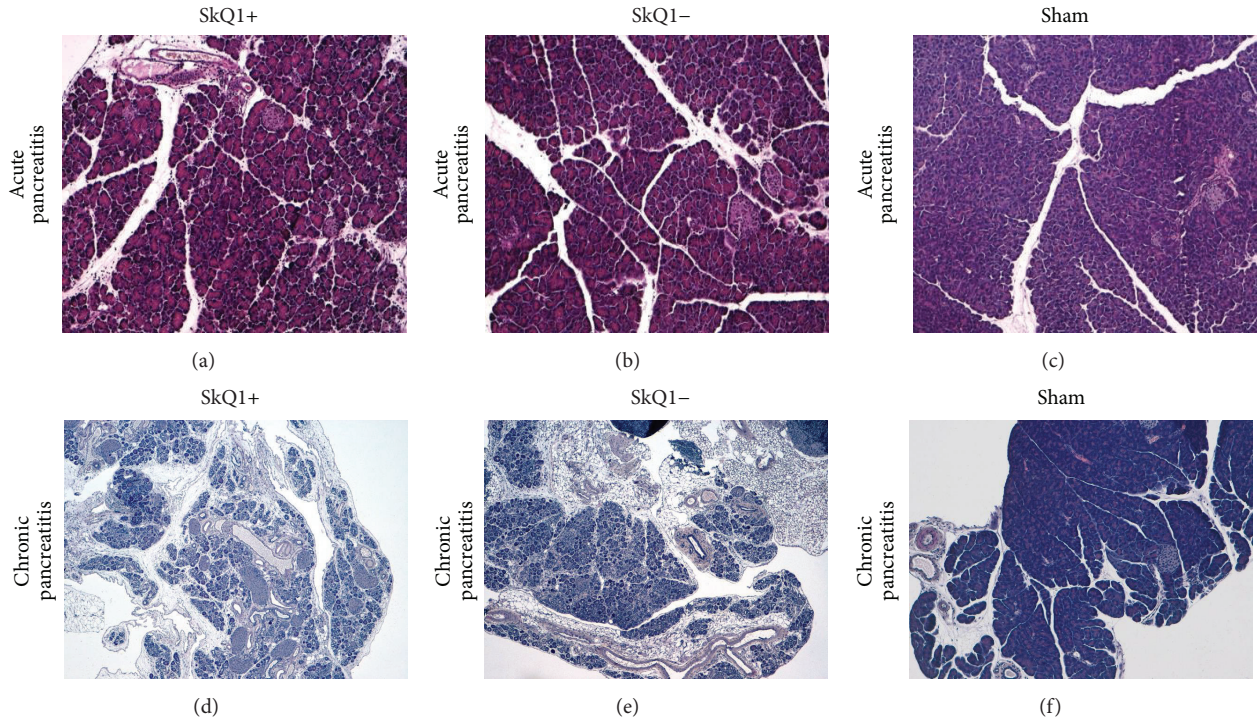

Figure 7: Representative HE staining of pancreatic tissue samples of mice with acute (a–c) and chronic pancreatitis (d–f).

acute pancreatitis [31, 32], which prompted numerous studies investigating the effect of antioxidants in acute and chronic pancreatitis [7]. One of the reasons for these disappointing previous results with antioxidants may be that they were only insufficiently able to penetrate mitochondria, one of the major sources for endogenous ROS production. To date, results are conflicting and, ultimately, do not point to a curative effect of antioxidants in pancreatitis [7, 8]. Nevertheless, mitochondria-targeted antioxidants have not been part of these investigations so far. Physiologically, ROS are byproducts of the mitochondrial oxygen metabolism [33] and are thought to interfere with apoptosis pathways [34]. In this respect, an imbalance of ROS and cellular antioxidative mechanisms has been linked to the pathogenesis and progression of acute and chronic pancreatitis. Mitochondria-targeted antioxidants have been shown to exert antioxidative effects at nanomolar concentrations inside mitochondria [23] and thus we hypothesized that scavenging ROS at their mitochondrial origin would reduce tissue injury in acute and chronic pancreatitis.

For acute pancreatitis, it has been shown that antioxidants may have a protective effect when administered as a pretreatment [5]. However, the results of the present study show that a pretreatment with SkQ1 does not result in reduced pain or tissue injury in cerulein-induced acute pancreatitis. The cerulein model for acute pancreatitis is the most frequently used model; however, it only induces a mild variant of acute pancreatitis over a very short time period of no more than 10 hours. It may, therefore, be possible that the influence of ROS and oxidative stress on pain and disease severity in acute pancreatitis is not as critical as initially hypothesized. SkQ1 exhibits a higher antioxidant activity than another structurally similar antioxidant—MitoQ [35]. Moreover, SkQ1 has in contrast to MitoQ a very wide therapeutic "window," in the order of 10^3, between its antioxidant and prooxidant effects [25]. Analogous to the results of the present study, Huang et al. demonstrated that the mitochondria-targeted antioxidant MitoQ does not improve tissue injury in a mouse model of acute pancreatitis [24]. Most interestingly, Huang et al. reported increased cell death rates in mice treated with MitoQ [24]. When comparing MitoQ to SkQ1, one has to consider that SkQ1 has a higher affinity to mitochondrial cardiolipin and has been demonstrated to quench ROS more effectively than MitoQ, and its antioxidant action was found to be exhibited even at nM concentrations [23, 25]. Taking these data and the results of Huang et al. [24] into account, a low, purely antioxidant concentration of 5 nmol/kg SkQ1 was considered sufficient in the present study. The same concentration has previously been shown to exert beneficial immunological effects in healthy [26] and pancreatic cancer bearing mice [26].

In chronic pancreatitis, pancreata of mice treated with SkQ1 display an even more severe disease when compared to untreated mice. In this respect, antioxidants have previously been described to hinder apoptosis and propel necrosis in pancreatitis [34]. Thus, a potential shift from apoptosis to necrosis might serve to explain increased tissue injury in experiments on chronic pancreatitis. Moreover, our results

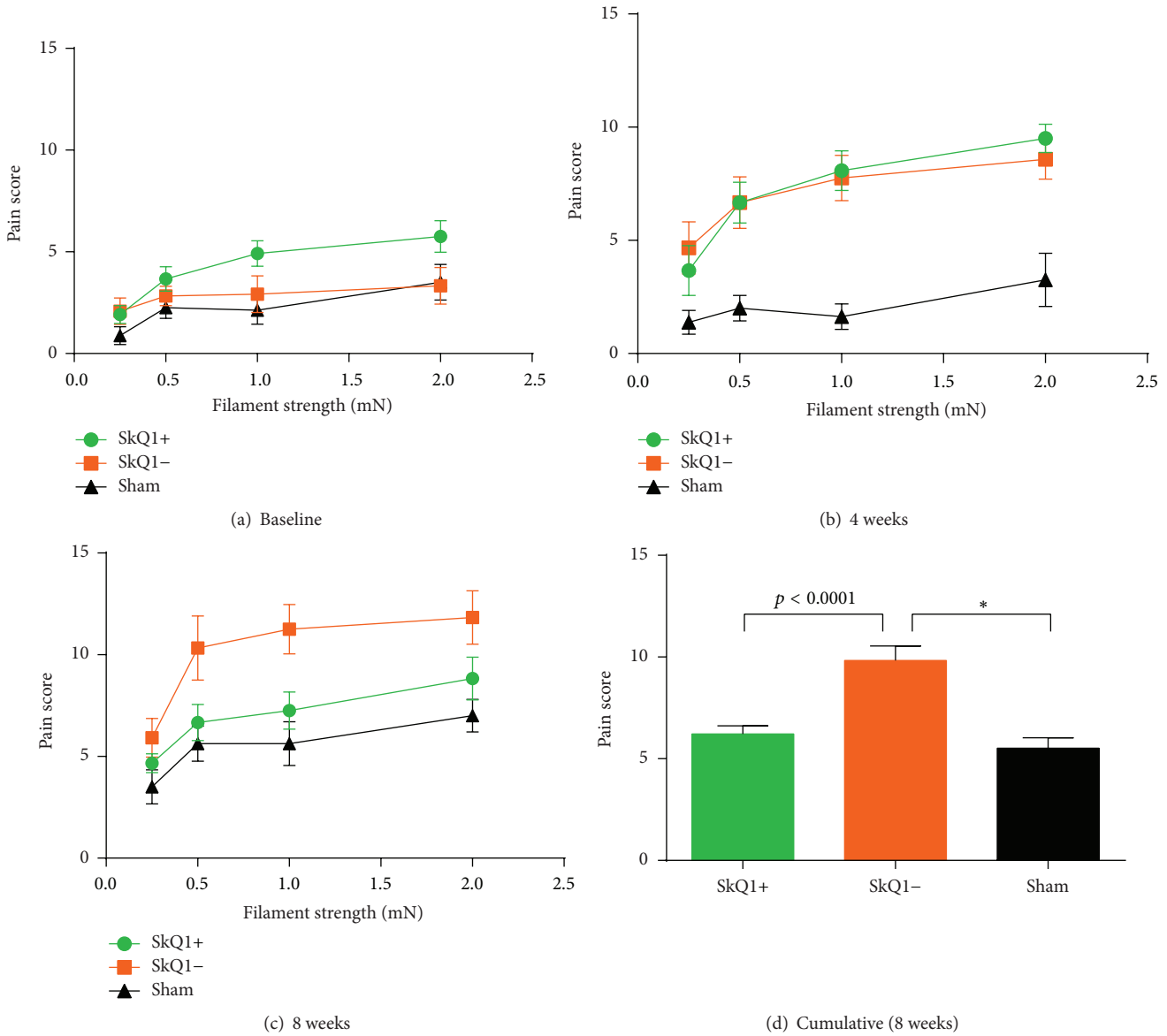

Figure 8: Evoked pain-related behavior measured by Von Frey's hairs in chronic pancreatitis: while baseline measurements did not differ significantly (a), mice with pancreatitis developed significantly increased pain scores after 4 weeks when compared to the saline controls (b). After 8 weeks of pancreatitis and 8 weeks of treatment, the SkQ1-treated mice show significantly less pain-related behavior than the untreated controls (c + d). *Statistically significant results ($p < 0.05$).

indicate that antioxidants do not only exert cytoprotective effects by scavenging ROS but rather interfere with a complex network of cellular messenger and effector proteins.

SkQ1 was not able to attenuate tissue injury in chronic pancreatitis, but mice treated with SKQ1 showed significantly less pain-related behavior. Among others, pain in chronic pancreatitis has been described as the result of peripheral neuropathy and neural damage [36, 37]. Furthermore, previous studies on mitochondria-targeted antioxidants clearly show neuroprotective effects of SkQ1 [16, 19]. We, therefore, hypothesize that SkQ1 treatment may be able to reduce the ROS-mediated intrapancreatic neural damage and thereby reduce pancreatic pain in chronic pancreatitis. In this respect, a recently published meta-analysis demonstrated a moderate pain reduction in chronic pancreatitis patients treated with antioxidants [38, 39]. Thus, an analgesic effect of SkQ1 in chronic pancreatitis may be postulated. However, this potential pain relief comes at the expense of increased tissue injury. The lack of parallelism between reduced pain and worsened tissue injury supports the hypothesis that the analgesic effect of SkQ1 is not mediated via inhibition of inflammatory processes within the pancreatic tissue. In this regard, amelioration of neural damage may be a possible mechanism. In pancreatic cancer, where pancreatic tissue injury is less important in advanced stages, mitochondria-targeted antioxidants might be an option as an auxiliary analgesic.

5. Conclusion

SkQ1 may aid in reducing pain in chronic pancreatitis. However, tissue injury in acute and chronic pancreatitis is not diminished by SkQ1. In the case of chronic pancreatitis, SkQ1 may increase disease severity. Thus, further studies are needed to identify the mechanism for increased tissue injury after SKQ1 in chronic pancreatitis and to elucidate the potential of SKQ1 as an analgesic in this setting.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Alexandr V. Bazhin and Jan G. D'Haese contributed equally.

Acknowledgments

The authors are pleased to acknowledge Ines Nachtigall and Karin Enderle for their excellent technical assistance. Additionally, the authors thank the Belozersky Institute of Physico-Chemical Biology from the Lomonosov Moscow State University for providing the SkQ1 solution. This project was financially funded by the "Förderprogramm für Forschung und Lehre (FöFoLe)" of the Ludwig Maximilians University Munich (Reg. no. 889).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4782426, 7 pages
http://dx.doi.org/10.1155/2016/4782426

Research Article

Oxidative Stress in Cancer-Prone Genetic Diseases in Pediatric Age: The Role of Mitochondrial Dysfunction

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Received 18 March 2016; Accepted 10 April 2016

Academic Editor: Gabriele Saretzki

Oxidative stress is a distinctive sign in several genetic disorders characterized by cancer predisposition, such as Ataxia-Telangiectasia, Fanconi Anemia, Down syndrome, progeroid syndromes, Beckwith-Wiedemann syndrome, and Costello syndrome. Recent literature unveiled new molecular mechanisms linking oxidative stress to the pathogenesis of these conditions, with particular regard to mitochondrial dysfunction. Since mitochondria are one of the major sites of ROS production as well as one of the major targets of their action, this dysfunction is thought to be the cause of the prooxidant status. Deeper insight of the pathogenesis of the syndromes raises the possibility to identify new possible therapeutic targets. In particular, the use of mitochondrial-targeted agents seems to be an appropriate clinical strategy in order to improve the quality of life and the life span of the patients.

1. Introduction

Reactive oxygen species (ROS) have crucial roles in many physiological and pathophysiological processes. A delicate balance between oxidants and antioxidants is essential for physiological functioning. On the contrary, the loss of this balance usually leads to dysfunctions and cellular damage at various levels, including membrane phospholipids, proteins, and nucleic acids [1–6].

In 1956 Harman postulated the free radical theory of ageing, according to which a redox imbalance and a ROS surplus are involved in the cellular damage that accompanies ageing and age-related diseases such as neurodegenerative diseases and cancer [7]. Since then, a huge body of literature has been produced on the role of oxidative stress (OS) in ageing and carcinogenesis, and a clear link between OS and the development of specific types of cancer has been ascertained [8–11]. In particular, the DNA damage inflicted by ROS contributes to the initiation and progression of carcinogenesis. ROS are able to react with DNA, damaging nitrogenous bases or producing double-strand breaks. They can also oxidize lipids and proteins, resulting in the production of intermediate species which in turn react with DNA. Several repair mechanisms intervene in removing DNA injuries; however, disrepair of DNA damage may occur in some cases, resulting in base substitutions or deletions leading to cancer development. In addition, DNA repair mechanisms have the tendency to decay with age: this leads to progressive accumulation of DNA injuries that accounts for the increased incidence of cancer with age [3, 12–15].

A second theory proposed to explain the mechanisms involved in ageing and in age-related diseases, including cancer, is the mitochondrial theory of ageing, postulated in 1984 by Miquel and Fleming and based on the presence of a mitochondrial dysfunction [16]. Increased ROS production, accumulation of damaged mitochondrial DNA (mtDNA), and progressive respiratory chain dysfunction are the three main principles of the theory. With age, a vicious cycle takes place: increased ROS production causes accumulation of oxidative damage in mtDNA, which is more sensitive to ROS-induced damage than nuclear DNA; mutated mtDNA codifies malfunctioning subunits of respiratory complexes that in turn

increase ROS production [17–20]. Signs of altered mitochondrial activity can be recognized in many OS related disorders, thus proving the existence of a strict connection between OS and mitochondrial dysfunction [21].

OS is a hallmark in several genetic diseases. In particular, evidence has been reported of an OS intervention in the pathogenesis of a number of cancer-prone genetic syndromes. In some of these diseases a mitochondrial dysfunction has also been demonstrated [22].

Taking into account the link between OS and carcinogenesis and the pivotal role exerted by mitochondrial dysfunction, the use of mitochondrial-targeted antioxidants and micronutrients might be a good clinical strategy to prevent cancer development in these syndromes.

2. Mitochondrial Dysfunction and Cancer Development: Mitochondrial-Targeted Antioxidants

Abnormalities in mitochondrial functions have been reported in several human pathologies, including cardiologic, haematologic, autoimmune, neurologic, and psychiatric disorders. One of the main lines of research in this respect investigates the link between mitochondrial dysfunction and cancer [21]. In cancer cells the increased ROS production is linked to mtDNA mutations and to alterations of the bioenergetics and the biosynthetic state of cancer cells [23]. Cancer cells show indeed several metabolic alterations, including increased fatty acid synthesis and glutamine metabolism, and an increased aerobic glycolysis [24, 25]; the latter feature is known as the "Warburg effect" and is thought to be due to defective mitochondria [26]. The switch towards aerobic glycolysis enables cancer cells to use glucose supplies for the biosynthesis of macromolecules, to support their rapid growth. ROS surplus can also determine the peroxidation of fatty acids in mitochondrial membranes: for example, the peroxidation of mitochondrial phospholipid cardiolipin leads to the formation of reactive aldehydes which in turn react with proteins and DNA [23]. Alterations of mitochondrial proteins are involved in mitochondrial dysfunctions characteristic of cancer cells. Moreover, dysfunctional mitochondria are able to modulate cell cycle, gene expression, metabolism, and cell viability [27].

In view of these findings, a supportive therapeutic approach based on the use of mitochondrial-targeted substances might be an appropriate strategy. A mitochondrial nutrient is an agent able to protect mitochondria from oxidative damage and to improve mitochondrial function by preventing generation of ROS, scavenging free radicals, and preventing oxidized inactive proteins accumulation. It can also repair oxidative damage by enhancing antioxidant defense systems [28–30]. A number of mitochondrial cofactors have been tested in several clinical trials to verify their potential benefits. Among them, the most studied are alpha-lipoic acid (ALA), coenzyme Q_{10} (CoQ_{10}), and L-carnitine. ALA is a dithiol compound, derived from octanoic acid, that is known as an essential cofactor for mitochondrial bioenergetics' enzymes. It is a natural antioxidant found in every cell of the body and it is able to trigger the mitochondrial pathway of apoptosis in cancer cells [31, 32]. CoQ_{10} is an endogenous lipid synthesized by the human organism and also introduced in small amounts through the diet. It is an electron acceptor and donor, and it may occur in an oxidized form (ubiquinone) and a reduced form (ubiquinol). It is important for the maintenance of mitochondrial homeostasis and the prevention of free radical production; in the form of ubiquinol, it also acts directly as a scavenger [33–35]. L-Carnitine (β-hydroxy-γ-trimethyl-ammonium butyric acid) is involved in mammalian lipid metabolism: it is required in the transport of activated fatty acids from the cytosol into the mitochondrial matrix, where β-oxidation takes place. In addition, it seems to take part in the repair of induced single-strand DNA breaks and in the protection of DNA from ROS [36–38].

3. Oxidative Stress and Mitochondrial Dysfunction in Cancer-Prone Genetic Diseases

A group of genetic diseases, including Down syndrome (DS), Ataxia-Telangiectasia (AT), Fanconi Anemia (FA), Bloom syndrome (BS), and Werner syndrome (WS), show OS and mitochondrial dysfunction as a phenotypic hallmark. These genetic disorders share, among other things, predisposition to cancer development and premature ageing.

AT is characterized by progressive neurodegeneration, immunodeficiency, oculocutaneous telangiectasias, endocrine abnormalities, high cancer incidence, genome instability, and hypersensitivity to ionizing radiation [39, 40]. The lifetime prevalence of cancer is about 40% [41]. In children with AT the most frequent cancer cases are acute lymphocytic leukemia and lymphoma [42]. AT is an autosomal recessive disorder caused by mutational inactivation of *ATM* gene, located on the long arm of chromosome 11. *ATM* gene encodes a protein belonging to the PI3/PI4-kinase family. The ATM protein is an important cell cycle checkpoint kinase involved in the repair response to DNA double-strand breaks [43, 44]. Loss of ATM function leads to genomic instability with chromosome breaks, translocations, and aneuploidy [45]. A link between OS and AT has been demonstrated in several studies [46–49]; recent research has provided some possible new mechanisms for oxidative damage associated with ATM deficiency that are independent of the DNA damage response pathway. In particular, ATM seems to be able to influence ROS production through the modulation of mitochondrial activity [50–52]. AT cells established from AT patients show an abnormal structural organization of mitochondria with a decreased membrane potential and an increased basal expression level of several nuclear DNA-encoded genes whose proteins are involved in oxidative damage response and are targeted to mitochondria. In addition, they show decreased overall mitochondrial respiratory activity: this activity could be rescued by treating the cells either with ALA or by the expression of full-length ATM, suggesting that the protein is required for the regulation of mitochondrial dysfunction [53]. In the light of these data,

the use of antioxidants directed at mitochondrial ROS could be a therapeutic strategy for AT patients. D'Souza et al. demonstrated that reducing mitochondrial ROS through overexpression of catalase targeted to mitochondria (mCAT) alleviates AT-related pathology in *ATM*-deficient mice, with particular regard to cancer pathology [54]. Berni et al. studied the effect of pretreatment with L-carnitine on DNA damage in normal and ATM-deficient cells established by AT patients and found that L-carnitine enhanced the rate and extent of DNA repair in AT cell lines; a reduction of all types of chromosomal aberrations was also observed [55].

FA is characterized by bone marrow failure leading to pancytopenia, physical abnormalities (including short stature, abnormal skin pigmentation, malformation of the thumbs, forearms, skeletal system, eyes, kidneys and urinary tract, ears, heart, gastrointestinal system, and central nervous system), type 2 diabetes mellitus, hypogonadism, and developmental delay [56]. Affected patients have an increased risk of malignancy, primarily acute myeloid leukemia. The risk of solid tumors is also increased [57]. FA is caused by mutation in 15 known genes whose functions are especially linked to DNA repair pathways [58–61]. There is a huge body of literature on the link between OS and FA, with numerous studies since the 1970s. The cells from FA patients show a prooxidant state and some of the genes linked to the syndrome's pathogenesis encode proteins involved in redox homeostasis. Moreover, FA patients display downregulation of major antioxidant defense genes [62–65]. The presence of mitochondrial defects in FA cells has been highlighted by recent literature: these defects seem to be directly connected to the increased ROS production and to the concurrent depletion of antioxidant defenses. In particular, FA cells show excess formation of mitochondrial ROS with a decreased mitochondrial membrane potential, decreased ATP production, impaired oxygen uptake, abnormalities in mitochondrial ultrastructure, and inactivation of mitochondrial activities involved in bioenergetics pathways and ROS detoxification [66–68]. Ponte et al. studied the protective effect of ALA and N-acetylcysteine (NAC) on chromosome instability in cells established from FA patients and found that the micronutrients cocktail is able to improve the genetic stability of FA lymphocytes *in vitro* [68]. A possible role for mitochondrial nutrients as chemopreventive agents in FA is suggested by these data. In this regard, a pilot study on the use of quercetin in children with FA has been set up by the Cincinnati Children's Hospital Medical Center and is currently recruiting participants. Primary outcome of the study is to assess the feasibility, toxicity, and pharmacokinetics of oral quercetin therapy in FA; secondary outcomes include assessment of the impact of quercetin on ROS reduction (Clinical Trial Identifier: NCT01720147). Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid with anti-inflammatory and antioxidant properties and seems also to enhance mitochondrial functionality [69, 70].

DS is one of the most common genetic anomalies. The main features of the syndrome are cognitive impairment, craniofacial dysmorphism, gastrointestinal abnormalities, congenital heart defects, endocrine abnormalities, neuropathology leading to dementia, and immunological defects. In approximately 95% of patients, DS is caused by full trisomy 21; the remaining cases are linked to mosaicism and translocations [71]. Affected children show a higher incidence of leukemia than the general population [72, 73]. DS seems to be characterized by a chronic prooxidant state: ongoing OS can be demonstrated from embryonic life and evidence of mitochondrial dysfunction has been reported, including alteration in the membrane potential, oxidative damage to mtDNA, ultrastructure changes such as abnormally shaped mitochondria, and diminished levels of microtubules [22, 74, 75]. Chromosome 21 contains several genes implicated in OS, above all Cu/Zn superoxide dismutase (*SOD1*). SOD1 is implicated in antioxidant defense: it catalyzes the dismutation of $^{\bullet}O_2^-$ to molecular oxygen (O_2) and H_2O_2, which can be converted by catalase and glutathione peroxidase to water. The triplication of chromosome 21 leads to an imbalance in the ratio of SOD1 to catalase and glutathione peroxidase, resulting in the accumulation of H_2O_2 [76, 77]. Tiano et al. evaluated the effect of CoQ_{10} administration to DS patients. At the beginning a mild protective effect on DNA was demonstrated at the cellular level, but the treatment failed to modify the overall oxidative damage at the patient level. After a longer follow-up and prolonged treatment, an age-specific reduction in the percentage of cells showing the highest amount of oxidized bases was highlighted, indicating a potential role of CoQ_{10} in modulating DNA repair mechanisms [78, 79].

BS is a rare, autosomal recessive disorder exhibiting numerous clinical features including sensitivity to sunlight, growth retardation, immunological disorders, and predisposition to cancer [80, 81]. Cells established by BS patients show excess DNA damage with a decreased glutathione disulfate : glutathione (GSSG : GSH) ratio [21, 82].

Finally, progeroid syndromes are a group of disorders characterized by clinical features mimicking physiological ageing at an early age. Several causative genes have been identified: genes encoding DNA repair factors (DNA helicases) and genes affecting the structure or posttranslational maturation of lamin A, which is a major nuclear component. Moreover, several animal models show abnormal mitochondrial function [83]. In particular, in WS involvements of the defective WRN protein in DNA stability and in redox balance have been observed and mitochondrial ultrastructure anomalies were found in cells from WS mouse model [21, 84].

4. Oxidative Stress and Beckwith-Wiedemann, Costello, and Prader-Willi Syndromes

BWS is a genomic imprinting disorder characterized by abdominal wall defects, macroglossia, pre- and postnatal overgrowth, neonatal hypoglycemia, visceromegaly, and increased risk of developing cancer in childhood, such as Wilms' cancer, hepatoblastoma, neuroblastoma, adrenocortical carcinoma, and rhabdomyosarcoma. The lifetime risk of developing cancer is approximately 7.5% [85]. The syndrome is associated with alterations in 2 distinct imprinting domains on 11p15: a telomeric domain containing the *H19* and *IGF2* genes and a centromeric domain including the *KCNQ1OT1* and *CDKNIC* genes. Disorders of imprinting in the telomeric

domain are associated with overgrowth and tumor development; imprinting defects at *KCNQ1OT1* are associated with the development of other embryonal tumors [85].

Costello syndrome is a rare genetic disease characterized by coarse facies, short stature, loose folds of skin on the hands and feet, severe feeding difficulties and failure to thrive, cardiac anomalies, developmental disability, and increased risk of malignancies, especially rhabdomyosarcoma, with an approximately 15% lifetime risk. The only gene currently known to be associated with the syndrome is Harvey rat sarcoma viral oncogene homolog (*HRAS*). Defects in this gene are implicated in a variety of cancers, including bladder cancer, follicular thyroid cancer, and oral squamous cell carcinoma [86]. The protein encoded by *HRAS* belongs to the Ras-mitogen-activated protein kinase (MAPK) pathway. Recent data demonstrated a functional connection between the Ras-MAPK pathway and mitochondrial function, and functional defects in mitochondrial respiration could be induced by oncogenic HRAS transformation, suggesting a possible role for mitochondrial dysfunction in the pathogenesis of CS [87–89].

By measuring a redox biomarker profile, the presence of a prooxidant state in patients affected by CS and BWS was documented. The administration of potassium ascorbate with ribose (PAR), which acts as antioxidant, determined a progressive decrease in OS biomarkers until their normalization, together with an improvement in the clinical conditions of the patients. No neoplastic disease was observed during a follow-up period of 10 years [90].

Potassium ascorbate is a salt derived from natural ascorbic acid; it is totally nontoxic and has antioxidant effects, combining the antioxidant action of vitamin C with the stabilizing intracellular effects of potassium. The ribose acts as a catalyst strengthening the action of potassium ascorbate [91, 92]. Ascorbic acid has been used in the prevention of cancer with promising results [93]. Mitochondria may be one of the principal targets of its activity: at higher concentrations vitamin C seems to increase ATP production by increasing mitochondrial electron flux and to induce apoptosis in cancer cells [94].

PAR supplementation gave promising results also in Prader-Willi syndrome (PWS), a genomic imprinting disorder whose most important feature is severe obesity leading to atherosclerosis and type 2 diabetes mellitus, in which a close relationship with OS has been widely demonstrated [95]. Interestingly, mitochondrial dysfunction was found in an imprinting center deletion mouse model of PWS, suggesting that an altered mitochondrial activity may contribute to the PWS pathogenesis [96]. Prader-Willi syndrome is not a cancer-prone disease; however, in recent years cases of early-onset cancer have been reported in PWS patients, probably due to the increased life expectancy, raising the question whether PWS predisposes to cancer development [97]. Indeed, there is evidence of a potential role of genomic imprinting and DNA methylation in human cancer [98, 99]. In addition, *Necdin* gene, which maps to chromosome 15q11–13, the region implicated in the pathogenesis of PWS, may have a potential tumor suppressor role, and it seems to be downregulated and hypermethylated or mutated in cancer [100, 101].

Antioxidant supplementation (PAR) in a PWS patient was associated with a progressive reduction of OS biomarkers occurring together with improvement in the clinical aspects of the patient, including the lack of development of the characteristic obesity [95]. Studies on relationship between oxidative stress and BWS, CS, and PWS, although being preliminary and based on a small group of patients, raise the prospect of future clinical trials based on larger case histories and with longer follow-up periods.

5. Conclusions

Oxidative stress is an important hallmark in several genetic diseases characterized by predisposition to tumor development and/or premature ageing. Studying the molecular mechanisms linking OS to the pathogenesis of these conditions allows identifying new possible therapeutic targets. Antioxidants administration to the affected patients might counteract their prooxidant state. Since a prooxidant state is often associated with mitochondrial dysfunction, the use of mitochondrial-targeted agents might be an appropriate clinical strategy in order to improve the quality of life and the life span of the patients.

Competing Interests

The authors declare that there are no competing interests.

Authors' Contributions

Perrone Serafina and Lotti Federica contributed equally to this work.

Acknowledgments

The authors thank EURAIBI (EURope Against Infant Brain Injury) Foundation for its partial grant.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4741694, 14 pages
http://dx.doi.org/10.1155/2016/4741694

Research Article

The Combination of α-Tocopheryl Succinate and Sodium Selenite on Breast Cancer: A Merit or a Demerit?

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Received 26 October 2015; Revised 5 February 2016; Accepted 7 February 2016

Academic Editor: Alexandr V. Bazhin

α-Tocopheryl succinate (α-TOS), a mitochondria-targeting agent, induces apoptosis in malignant cells in vitro and in vivo. Selenite is a nutritional supplement that has been shown to stimulate apoptosis in cancer cells. This study was designed to investigate the cytotoxic effect of combined treatment of α-TOS and sodium selenite (SSe) in vitro and in vivo and to explore their effect on apoptosis and autophagy in breast cancer. The type of interaction between α-TOS and SSe was evaluated and levels of oxidative stress and apoptotic and autophagic markers were determined. SSe alone showed varying degrees of cytotoxicity on all the tested cell lines. Its combination with α-TOS was antagonistic in vitro in MCF7 and in vivo in mice bearing Ehrlich tumor compared to α-TOS-treated one. Combination of TOS with 2 μM of SSe increased the level of glutathione without changes in antiapoptotic markers Bcl-2 and Mcl-1 at 16 and 48 hrs. SSe decreased caspase 3 activity and protein level of caspases 7 and 9, while it increased autophagic markers beclin-1 and LC3B protein levels of MCF7 cells treated with α-TOS. In conclusion, SSe antagonizes α-TOS-induced apoptosis via inhibition of oxidative stress and promoting prosurvival machinery of autophagy.

1. Introduction

Breast cancer is the second most common cancer in the world and the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012. It is the most common cancer in women both in more and in less developed regions [1]. Despite modern approach in improving patient compliance by combating breast cancer with more selective and less toxic drugs, treatment strategies remain a challenge. α-Tocopheryl succinate (α-TOS) is a provitamin known to reduce the growth of various cancer cell lines, by virtue of its prooxidant effect, including breast, prostate, pancreatic, and melanoma cancers, while shielding normal cells [2–5]. In addition, in vivo, α-TOS causes suppression of chemically induced forestomach cancer [6] and inhibits the growth of several inoculated cancer cells [7, 8].

Modulation of signaling pathways by micronutrients is a promising strategy for cancer prevention and treatment. It was demonstrated that combination of polar carotenoids is more effective against the growth of tumor cells in vitro than the individual agents [9]. Selenium is an essential dietary micronutrient for all mammalian species. Selenium-replete diets are thought to result in maximal expression of selenoproteins [10], a family of proteins whose functions include antioxidant activities and maintaining the intracellular redox state [11]. Sodium selenite (SSe) exhibits greater toxicity towards malignant than benign cells [12] and inhibits the development of mammary tumors in a rat model [11]. Selenite-mediated generation of the superoxide radical anion ($O_2^{\bullet}$) is thought to cause oxidative stress, leading to cellular damage and death. The $O_2^{\bullet}$ observed in selenite-treated cells may originate from mitochondria [13]. SSe was reported to induce DNA damage, particularly DNA strand breaks. Furthermore, it may sensitize malignant cells to apoptosis induced by other antineoplastic treatment modalities, thereby improving the efficacy and outcome of potential antineoplastic therapy [14]. Hence the combination of α-TOS with SSe may have superior antitumor activity to α-TOS alone. Therefore, the aim of this study was to test the combined antitumor activity of α-TOS and SSe via assessing some apoptotic and autophagic markers.

2. Materials and Methods

2.1. Human Cancer Cell Lines. In this study, a panel of the available cell lines were tested for their chemosensitivity to either α-TOS or SSe. Two concentrations (nutritional 2 μM and supranutritional 10 μM) of SSe were used in combination with different concentrations of α-TOS, and type of drug interaction was evaluated for all the tested cell lines. The most sensitive cell line to the treatment regimen was selected and subjected to further investigations to explore the mechanism of this interaction. Breast adenocarcinoma MCF7, cervical adenocarcinoma HeLa, lung carcinoma A549, mammary gland ductal carcinoma T47D, prostate adenocarcinoma PC3, hepatocellular carcinoma HepG2, and colorectal carcinoma HCT-116 cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA) and maintained at the National Cancer Institute in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin and routinely incubated with 5% CO_2 in a humidified atmosphere at 37°C.

2.2. Cytotoxicity Assay. The cytotoxicity assay was carried according to the method described by Skehan et al. [15]. Cells were seeded in a 96-well plate at density of 3×10^3 cells/well and were incubated overnight at 37°C in humidified 5% CO_2 incubator. Cells were treated with 20–100 μM α-TOS (Sigma Aldrich, USA) and with 2–10 μM SSe (Sigma Aldrich, USA) for 48 and/or 72 hours. Control cells were treated with the RPMI-1640 medium (Biowest, France) containing 0.1% DMSO. A combination regimen was designed using the following regimens:

(i) *Ist Combination Regimen*. Fixed 2 μM SSe with 20–100 μM α-TOS concentrations.

(ii) *2nd Combination Regimen*. Fixed 10 μM SSe with 20–100 μM α-TOS concentrations.

After the desired time intervals, the cells were fixed with 20% trichloroacetic acid (Sigma Aldrich, USA), washed, and stained with 0.4% sulforhodamine-B dye (Sigma Aldrich, USA). The produced color was measured spectrophotometrically at 575 nm using ELISA plate reader (Tecan Sunrise™, Germany).

2.3. Evaluation of Drug Interaction. The degree of interaction between the two drugs was calculated using the combination index, according to the isobologram equation [16]: $CI = d1/D1 + d2/D2$, where $d1$ and $d2$ represent the concentrations of α-TOS and SSe that, when given in combination, produce a specific response, and $D1$ and $D2$ represent the concentrations of α-TOS and SSe that, when given individually, produce the same effect. When combination index values are less than 1 ($CI < 1$) they indicate synergism, while $CI = 1$ represents additivity and $CI > 1$ indicates antagonism.

2.4. Determination of α-TOS Uptake by MCF7 Cells. MCF7 cells 10×10^3/well were seeded in colorless RPMI-1640 medium and left for 24 hours. The plate was divided into 2 groups as follows:

(a) Group I: treated with 60 μM α-TOS.

(b) Group II: treated with 60 μM α-TOS and 2 μM SSe.

The medium was then aspirated after 0-, 2-, 4-, 6-, and 24-hour intervals and centrifuged and the supernatant was stored at −20°C till HPLC assay.

2.4.1. Sample Extraction and Preparation. 100 μL of the medium was mixed thoroughly with 1.4 mL acetonitrile (Alliance Bio, USA) and centrifuged at 10 000 rpm for 10 minutes at 4°C. Twenty microliters of the resulting supernatant was then injected into an HPLC system consisting of 520 pump gradient, 560 autosampler, and 535 spectrophotometric detector (Bio-Tek, Italy). The analytical column used was Equisil ODS (250 mm × 4.6 mm ID, 10 μm). The mobile phase consisted of acetonitrile and water (90 : 10 v/v) and flow rate of 2 mL/min, with ultraviolet detection at 205 nm [17]. Results are expressed as μM α-TOS after calibration with standard curve (10–60 μM) for α-TOS.

2.5. Determination of Total Lipid Peroxides Content (Measured as Malondialdehyde (MDA)). Lipid peroxidation products were quantified by measuring MDA level [18]. Treated and control cells were mixed well with 20% (w/v) trichloroacetic acid (TCA) containing 0.8% (w/v) thiobarbituric acid (TBA) (Sigma Aldrich, USA), incubated in a boiling water bath for 1 hour. The absorbance of the supernatant was determined at 535 nm using a spectrophotometer (Spectronic, Milton Roy Co., USA). The concentrations were calculated using MDA standard calibration curve by preparing serial dilutions of 1,1,3,3-tetraethoxypropane (Sigma Aldrich, USA).

2.6. Determination of Non-Protein SH (GSH) Content. Reduced glutathione was determined adopting Ellman's method [19]. MCF7 cells were harvested as previously mentioned, protein was precipitated with trichloroacetic acid, and Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] (DTNB) (Sigma Aldrich, USA) was added to clear supernatant. The absorbance was read at 405 nm and total SH was calculated as μM of GSH/mg protein.

2.7. Western Blot. The method was carried out according to Maniatis et al. [20]. Treated and control cells were incubated with lysis buffer [150 mM NaCl (Riedel-deHaën, Germany), 10 mM Trizma (MP Biochemical, France), 0.2% triton X-100 (MP Biochemical, France), 0.3% NP-40 (Fluka BioChemika, Switzerland), and 0.2 mM sodium orthovanadate (Sigma Aldrich, USA)] for 30 minutes on ice. Cells were homogenized by repeated sonication and vortex for 30 seconds and centrifuged at 14000 g for 15 minutes at 4°C. The supernatant was collected and the protein concentration was determined. Protein was separated by 10% SDS-PAGE and electroblotted onto PVDF membrane with primary mouse anti-human caspase 9 mAB (1 : 1000) (eBioscience, Austria), caspase 7 mAB (1 : 500) (Novus Biologicals, USA), Bcl-2 mAB (1 : 2000) (Sigma Aldrich, USA), Mcl-1 mAB (1 : 500) (R&D, USA), beclin-1 mAbs (1 : 500) (R&D, USA), and rabbit anti-human LC3B oligoclonal AB (1 : 500) (Invitrogen, USA), β-actin mAb (1 : 1000) (R&D, USA). The protein bands were visualized using Amersham™ ECL Western Blotting Detection Reagents on X-ray film (Fujifilm, Tokyo, Japan) after incubation of the membrane with the appropriate

secondary goat anti-mouse IgG or secondary goat anti-rabbit IgG antibodies (Sigma Aldrich, USA). Images were acquired with a scanner and analyzed with Scion Image Beta 4.0.2 (Scion Co., Frederick, MD, USA) software.

2.8. Determination of Enzymatic Activity of Caspase 3 in Cell Lysate. Caspase 3 activity was determined in cell lysate using caspase 3 activity colorimetric assay kit purchased from R&D, USA, according to the method described by Casciola-Rosen et al. [21]. Cells were harvested after exposure to different treatment regimens and caspase 3 activity was measured in accordance with the manufacturer's instructions. The results were expressed as fold increase in caspase activity of apoptotic cells over that of nonapoptotic cells.

2.9. Detection of Acidic Vesicular Organelles. Autophagy was detected using the lysosomotropic agent, acridine orange (Sigma Aldrich, USA) [22]. Treated and nontreated MCF7 cells were incubated with medium containing 1 μg/mL acridine orange for 10 minutes. The micrographs were taken using an inverted fluorescent microscope equipped with digital camera (NIKON, Japan) and supplied with blue (excitation BP 450–490) and green filters (excitation BP 510–550).

2.10. Determination of Human VEGF in Culture Medium by ELISA Kit. The culture medium of treated and control cells was aspirated and centrifuged at 10 000 rpm at 4°C for 10 minutes and the resultant supernatant was used for determination of VEGF by an ELISA kit (RayBio, USA) in accordance with the manufacturer's instructions [23]. The amount of VEGF was expressed as pg/mL.

2.11. Protein Assay. Protein content was determined in whole cell lysate according to the method described by Bradford [24] following the manufacturer's instructions of protein assay kit (Pierce Biotechnology, USA). Absorption was read at 595 nm with a spectrophotometer.

2.12. Assessment of the Antitumor Activities of α-TOS, SSe, and Their Combination. To explore the effect of this combination in vivo, we used the available model of animal bearing tumor [mice bearing Ehrlich ascites carcinoma (EAC)]. Female mice were transplanted subcutaneously in the right thigh with EAC cells (2×10^6) till reaching a palpable tumor mass (100 mm^3). The mice were divided into 4 groups (each group contained six mice) and injected intraperitoneally (i.p.) twice, every third day as follows:

Gr 1: injected with DMSO (0.1 mL/20 gm) and used as control.

Gr 2: injected with α-TOS (150 mg/kg) with a total dose of 300 mg/kg.

Gr 3: injected with SSe (0.5 mg/kg) with a total dose of 1 mg/kg.

Gr 4: injected with a simultaneous combination of α-TOS and SSe at the aforementioned doses.

The change in tumor volume was measured every other day using a caliper and calculated according to the following formula [25]:

$$\text{Tumor volume } \left(\text{mm}^3\right) = 0.52 \times A^2 \times B^2, \quad (1)$$

where A, B refer to the minor and major tumor axis, respectively.

2.13. Assessment of the Oncolytic Activities of α-TOS, SSe, and Their Combination. Female mice were injected i.p. with EAC. Twenty-four hours after cell inoculation, the mice were divided into 4 groups (each group contained ten mice), injected i.p. on 2 consecutive days with the aforementioned treatment regimens. The animals were observed daily and the percent survival, mean survival time (days), percent change in animal and body weight, and percent change in life span (% CLS) were calculated: % CLS = $(T - C/C) \times 100$, where

T = *average life span of treated mice*,

C = *average life span of control mice*.

2.14. Statistical Analysis. Unpaired t-test was used to compare two different treatment groups. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for post hoc analysis. Statistical significance was acceptable at a level of P value < 0.05. Graphs were performed using Prizm software program (GraphPad Prism software, version 5).

3. Results

3.1. SSe Antagonizes α-TOS Cytotoxicity in All the Tested Cell Lines. Results of the present study revealed that all the tested cell lines were resistant to concentrations of SSe that were used in this study (2–10 μM), IC_{50} > 10 μM except MCF7 which showed IC_{50} at 5.45 μM, Table 1. Moreover, when the cell lines were exposed to 20–100 μM of αTOS IC_{50} was >100 μM in Hela and T47D, and the cell lines IC_{50} ranged from 77 to 100, while in MCF7 it was 57.5 μM after 48 hours; data are shown in Supplementary Material (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/4741694). Treatment of all the cell lines, with combination of either 2 or 10 μM of SSe and different concentrations of αTOS, resulted in antagonistic effect, Table 1. Therefore MCF7 cell line was chosen, as it is the most sensitive cell line that showed growth inhibition to each drug alone.

3.2. SSe Antagonizes α-TOS Cytotoxicity in MCF7. Treatment of MCF7 cells with α-TOS or SSe alone resulted in decrease in cellular growth after 48 and 72 hours, Figures 1(a) and 1(b). The effect of combination of α-TOS with either 2 or 10 μM SSe was antagonistic on MCF7 cell line after 48 and 72 hours (Figures 1(c), 1(d), 1(e), and 1(f)). Therefore, we used the nontoxic concentration of SSe (2 μM) to study the mechanism of this antagonistic effect between α-TOS and SSe on MCF7 cells following 16 and 48 hours.

Table 1: Screening of cytotoxic effect of SSe (2–10 μM) and α-TOS (20–100 μM) and their combination with 2 μM and 10 μM SSe and the resultant combination indices after 48 hours on MCF7 cells. The results are obtained from 5 independent experiments performed in triplicate.

Human cancer cell lines	The 50% inhibitory concentration				Combination index (CI)	
	TOS (μM)	SSe (μM)	TOS + 2 μM SSe	TOS + 10 μM SSe	TOS + 2 μM SSe	TOS + 10 μM SSe
HeLa	>100	>10	>100	>100	1.05	2.37
A549	82	>10	90.5	>100	1.86	2.19
T47D	>100	>10	>100	>100	1.42	2.24
PC3	>100	>10	>100	>100	1.50	2.38
HepG2	83.3	>10	93.2	98.2	2.08	3.07
HCT-116	76.8	>10	89.8	91.3	1.25	2.22
MCF7	57.5	5.45	65.5	12.5	1.50	2.09

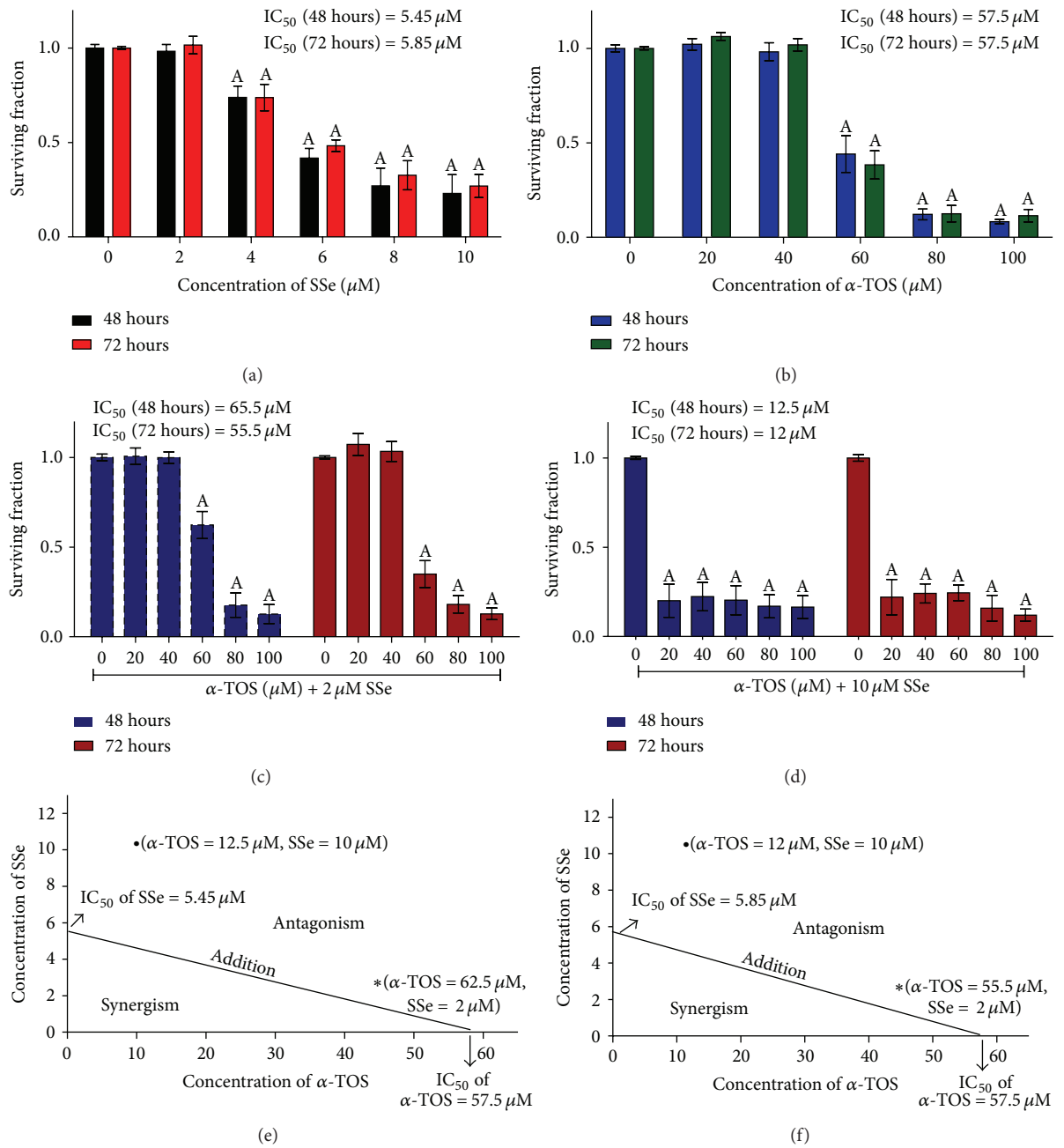


Figure 1: Surviving fraction of MCF7 cells treated with (a) SSe (2–10 μM) and (b) α-TOS (20–100 μM) and its combination with (c) 2 μM and (d) 10 μM SSe and their combination indices are represented in the isobologram after (e) 48 and (f) 72 hours. The results are expressed as mean ± SD of 5 independent experiments performed in triplicate. Statistical significance of results was analyzed by one-way ANOVA using Tukey's multiple comparison test. "A" significantly different from its respective control at $P < 0.05$.

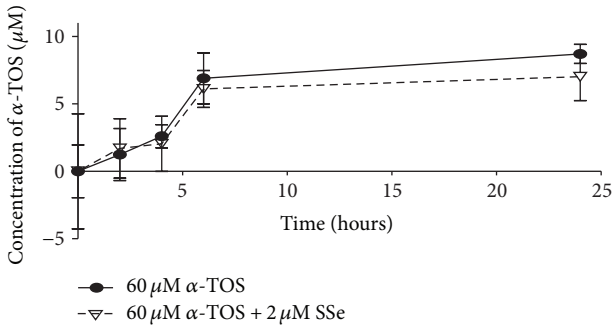


Figure 2: Effect of SSe on cellular uptake of α-TOS from the culture medium after different time intervals (0, 2, 4, 6, and 24 hours). Results are expressed as means ± SD of 3 independent experiments. Results were found nonsignificantly different at $P < 0.05$ using unpaired t-test.

3.3. SSe Does Not Affect Cellular Level of α-TOS. SSe did not affect significantly the cellular uptake of α-TOS at any point of the studied time (Figure 2).

3.4. SSe, at Nutritional Concentration, Acts as an Antioxidant While, at Super Nutrition Concentration, It Acts as Prooxidant. α-TOS produced a significant increase in MDA level after 16 hours, followed by a recovery to control value after 48 hours. On the other hand, low concentration of SSe (2 μM) significantly decreased MDA levels after 48 hours, whereas higher concentration of SSe (10 μM) significantly increased MDA level after both time intervals (Figures 3(a), 3(c), 3(e), and 3(g)). Regarding GSH level, it was shown that SSe (2 μM) alone produced significant increase in GSH by 12% and 46% at 16 and 48 hrs, while SSe at 10 μM concentration significantly reduced GSH level to 47.7% and 66% at 16 and 48 hrs, respectively. In addition, GSH level was significantly increased following exposure of cells to the combination of α-TOS and 2 μM SSe (Figures 3(b) and 3(d)), while combination with 10 μM SSe significantly decreased GSH level (Figures 3(f) and 3(h)).

3.5. α-TOS, SSe, and Their Combinations Do Not Affect Bcl-2 and Mcl-1 Protein Levels. Using scion image to precisely measure the protein level, it was found that the antiapoptotic protein levels of Bcl-2 or Mcl-1 did not change with any treatment regimen for both time intervals used (Figures 4(a), 4(b), 4(c), and 4(d)).

3.6. Either α-TOS or SSe Individually Increases Caspases, While Their Combinations Decrease Them. Both α-TOS and SSe resulted in activation and cleavage of caspase 9 and caspase 7 proteins as well as a significant increase in caspase 3 activity, following incubation for 16 and 48 hours compared to the control. Interestingly, the combination resulted in a significant inhibition of the activation of caspase 7 and activity of caspase 3 leading to inhibition of apoptosis (Figures 4(e), 4(f), 4(g), and 4(h)).

3.7. α-TOS, SSe, and Their Combinations Increase the Expression of Autophagic Proteins. SSe either alone or combined with α-TOS induced autophagy after both 16 and 48 hours of exposure. This was shown by an increase in beclin-1 and LC3B protein levels after both time intervals. On the other hand, α-TOS induced an early autophagy process, where the levels of both proteins were increased after 16 hours, but there was a recovery in LC3B level within 48 hours (Figures 5(a), 5(b), 5(c), and 5(d)). To determine the interplay between autophagy induction by SSe and apoptosis, cells were pretreated with chloroquine (CQ), an inhibitor of autophagy, prior to treatment with various concentrations (2–10 μM) of SSe for 48 hours. The IC_{50} was shifted to be 1.73 μM, indicating that inhibition of autophagy increased the apoptotic effect (Figure 5(e)). This was further elucidated by AO staining of cytoplasmic AVOs, which were detected after 48 hours in the cytoplasm of SSe and combination-treated cells but not in the α-TOS-treated cells (Figure 5(f)).

Table 2: Effect of administration of α-TOS (300 mg/kg), SSe (1 mg/kg), and their combination on the mean survival time (MST) and percentage change in life span (CLS) in EAC-bearing mice.

Treatment (mg/kg)	MST ± SD	CLS (%)
Control	12.8 ± 4.5	—
α-TOS	15.0 ± 5.2	17.2
SSe	18.1 ± 5.6	41.4
α-TOS + SSe	13.4 ± 6.7	3.9

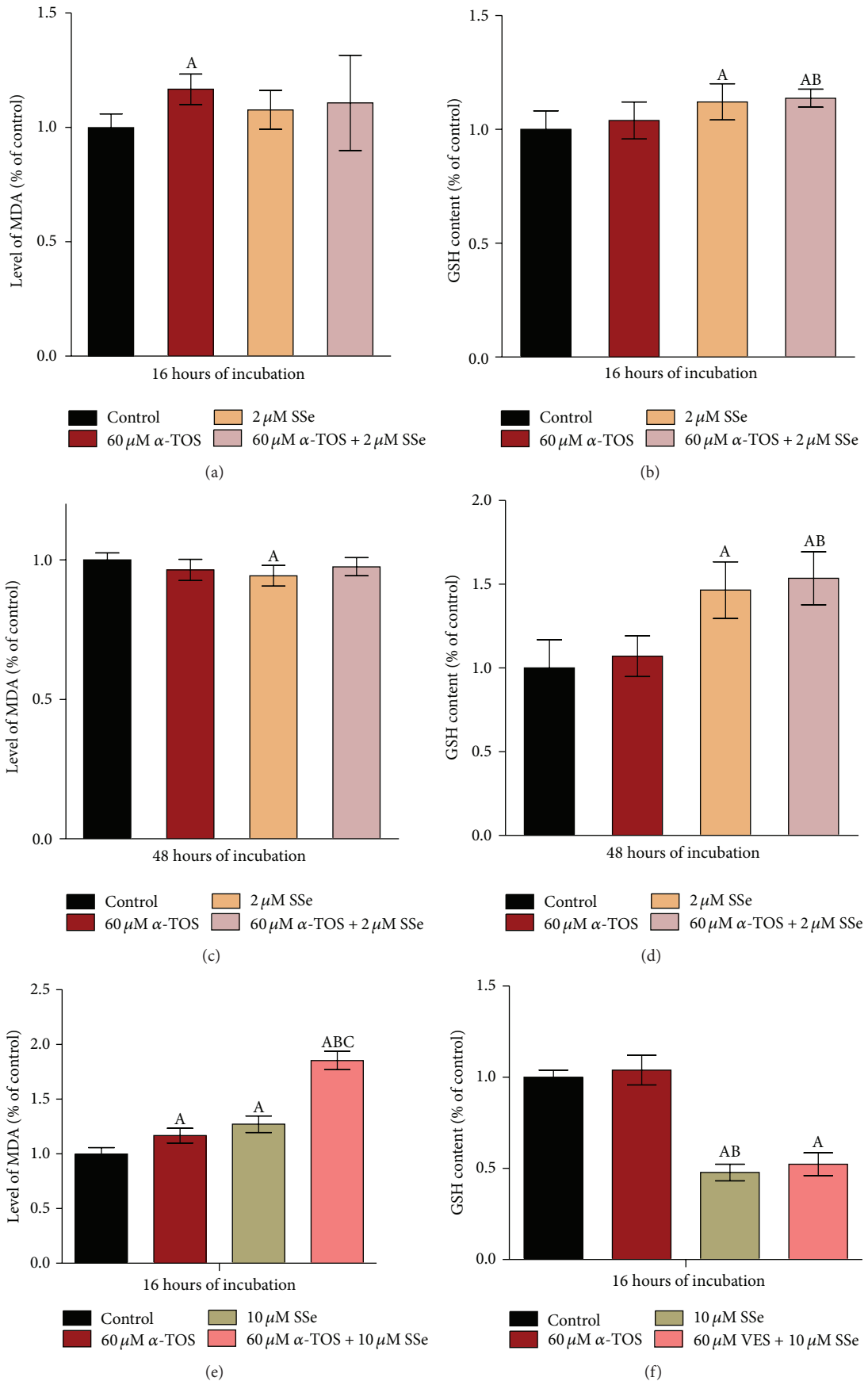
3.8. α-TOS and Its Combination with SSe Inhibit the Release of Human VEGF. The results revealed that secretion of VEGF into culture medium was significantly decreased in α-TOS-treated group after both time intervals, whereas SSe exposure for either time interval showed no significant change in its level as compared to control. Moreover, the combination of SSe with α-TOS inhibited VEGF release with the same efficacy as α-TOS (Figures 6(a) and 6(b)).

3.9. α-TOS, SSe, and Their Combinations Decrease Tumor Volume In Vivo. The volume of solid tumor in untreated control reached a size of 860 mm^3 7 days from tumor inoculation. However, it reached 266 mm^3 and 220 mm^3 7 days from tumor inoculation following treatment with α-TOS and SSe, respectively, while the combined treatment resulted in a tumor volume of 431 mm^3 which is significantly larger than α-TOS only (Figure 7(a)).

3.10. SSe Abrogates the Oncolytic Activity of α-TOS. Regarding the percent survival of mice, on day 18, none of the control tumor-bearing mice were alive, on day 23, none of the α-TOS-treated mice were alive, and on day 29 none of the SSe-treated mice were alive. Concerning the combination, on day 22, none of the mice were alive. Also, α-TOS, SSe, and their combination increased the life span of mice by 17.2, 41.4, and 3.9%, respectively (Table 2 and Figures 7(b) and 7(c)).

4. Discussion

In the present study, α-TOS inhibited the proliferation of MCF7 cells, with an early significant increase in MDA.



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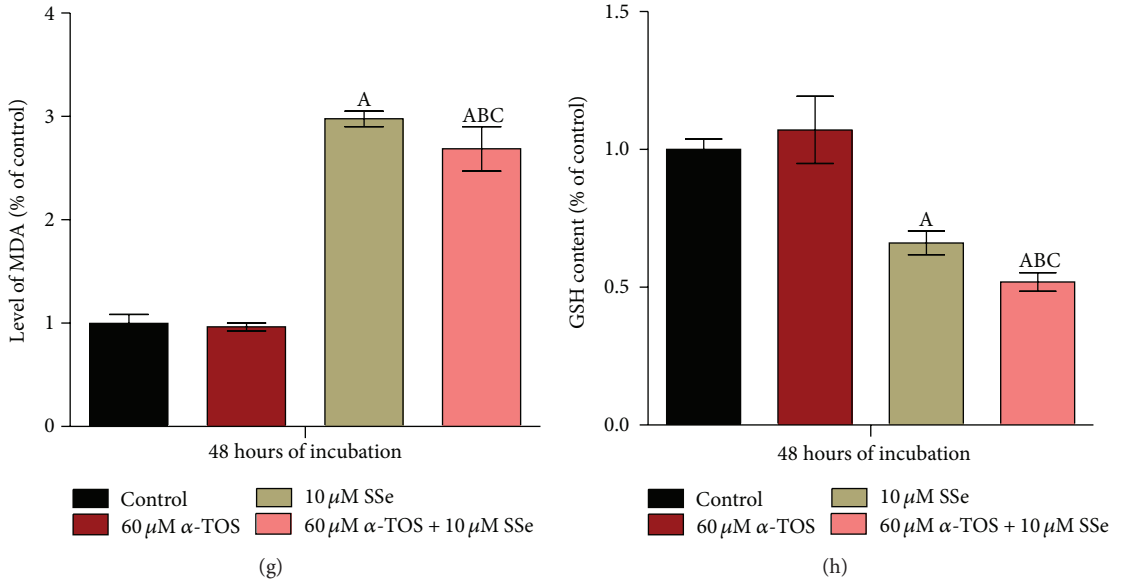
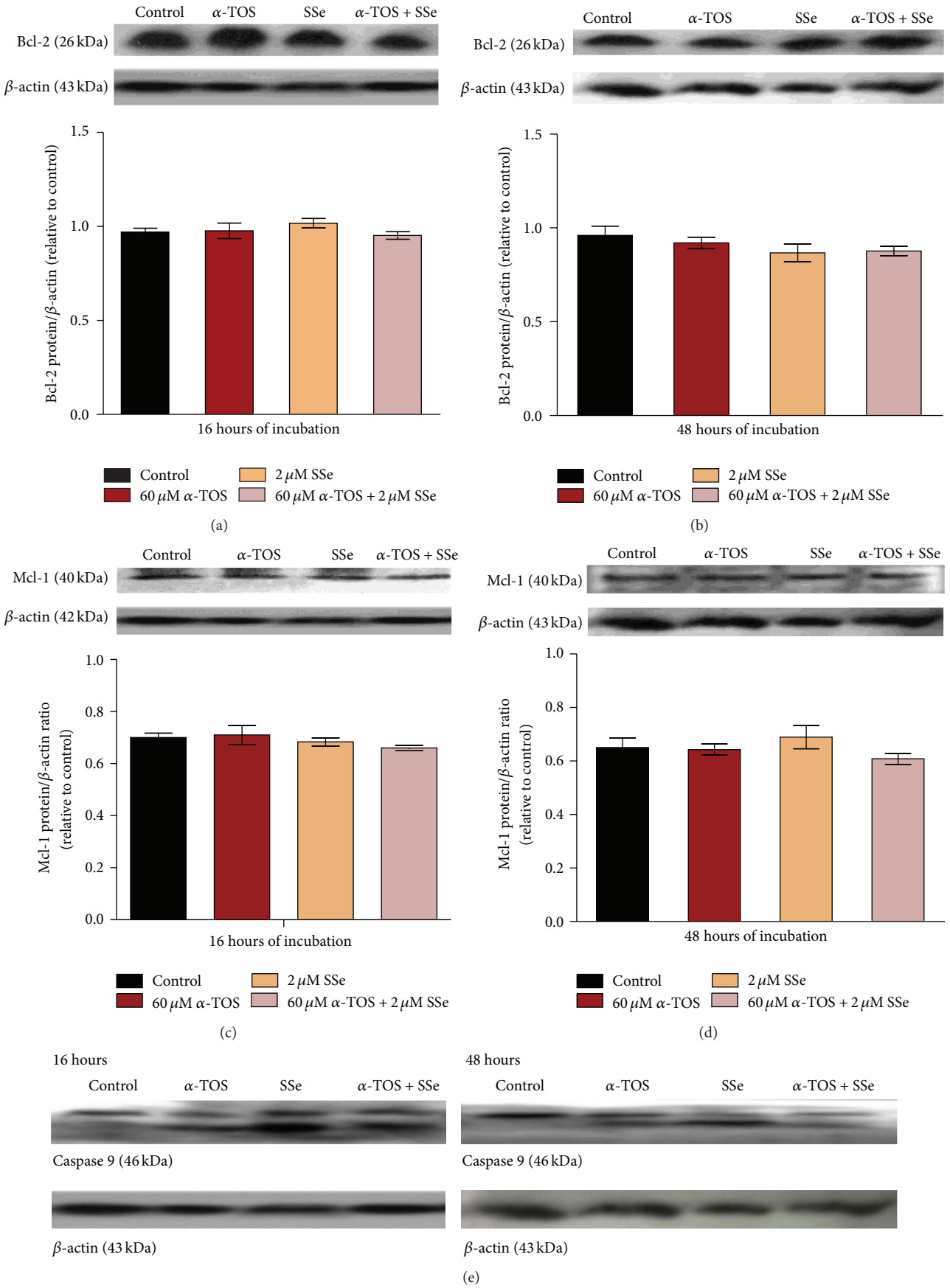


FIGURE 3: Combined effect of 60 μM α-TOS and 2 μM SSe on lipid peroxidation after (a) 16 hours and (c) 48 hours and on reduced glutathione levels after (b) 16 hours and (d) 48 hours. Combined effect of 60 μM α-TOS and 10 μM SSe on lipid peroxidation after (e) 16 hours and (g) 48 hours and on reduced glutathione levels after (f) 16 hours and (h) 48 hours in MCF7 cells. Results of MDA and rGSH are expressed as means ± SD of 5 independent experiments ($n = 15$ for MDA and $n = 12$ for rGSH). Statistical significance of results was analyzed by one-way ANOVA using Tukey's multiple comparison test. "A" significantly different from the respective control at $P < 0.05$; "B" significantly different from respective α-TOS at $P < 0.05$; "C" significantly different from respective SSe at $P < 0.05$.

Similar studies reported antitumor activity for α-TOS on different cancer cell lines, including prostate cancer [26], gastric cancer [27], pancreatic cancer [4], resistant mesothelioma [28], and HER2 overexpressing breast cancer cell line [29]. This cytotoxicity was convoyed by an early buildup of ROS, upon exposure to α-TOS in Jurkat cells [30], breast cancer cells [29], melanoma cells [31], prostate cells [32], and non-small cell lung cancer cells [33]. As a member of the mitocans, α-TOS disrupts the mitochondrial membrane potential causing the generation of ROS resulting in apoptosis [34]. α-TOS induced activation of caspases 7 and 9 and increased activity of caspase 3 without changes in the expression of antiapoptotic protein levels (Bcl-2 and Mcl-1) of MCF7 cells in our study. However, Gu et al. [35] found dramatic decrease in Bcl-2 protein level at 6 hours followed by a slight recovery at 12 hours suggesting metabolic degradation of α-TOS upon prolonged incubation. Kang et al. [33] found that cytotoxicity induced by α-TOS was cell type dependent. It was abrogated by prior addition of antioxidants, explaining the role of ROS in α-TOS-induced apoptosis. However, it was described that incubation of glioblastoma cancer cells with α-TOS resulted in apoptosis with negligible effects on ROS. Moreover, the presence of an antioxidant did not alter the rate of cell death. Moreover, ROS have been copiously reported as early inducers of autophagy upon nutrient deprivation. In addition, it is an evolutionarily conserved catabolic process, responsible for the routine degradation of bulk dysfunctional proteins and organelles [36]. Autophagy plays a protective role in response to a majority of anticancer drugs and in the pathogenesis process [36]. In the current study, we found that α-TOS produced early induction of autophagy manifested by increased beclin-1 protein level and an early increase in the expression of LC3B protein, responsible for the completion of the autophagosome formation, which recovered after prolonged incubation to control value. Likewise, Neuzil et al. [37] reported early or initiating lysosomal destabilization event in apoptosis induced by α-TOS that precedes both caspases activation and phosphatidyl serine externalization. They suggest that the key player in apoptosis was cathepsin D and cathepsin D-deficient cells showed lower caspase 3 activity and resist apoptosis.

Complementary therapies including dietary supplements, herbs, and vitamins play a major role in cancer prevention if utilized properly; they can change the course of cancer progression. Many dietary factors affect the rate of growth of cancerous tumors and specific dietary interventions may potentially reverse tumor progression. Results of this study showed inhibition of growth of MCF7 cells exposed to SSe. Low concentration of SSe significantly increased level of antioxidant glutathione, while higher concentration produced significant increase in ROS. Similarly, the anticancer activity of SSe was reported in previous studies on osteosarcoma [38], malignant mesothelioma [12], prostate cancer [39], and lung cancer cell lines [40]. The effect of SSe on ROS in different cell lines is controversial. In harmony with our data, Chatzakos et al. [41] and Fu et al. [42] reported that low concentrations of SSe can regulate cellular redox levels, resist peroxidation, and protect against cancer, while higher concentrations exert an oxidative stress resulting in ROS-mediated apoptosis. Sarada et al. [43] reported that addition of SSe to neuroblastoma cells in culture prior to hypoxia-induced ROS decreased the hypoxia-induced cell



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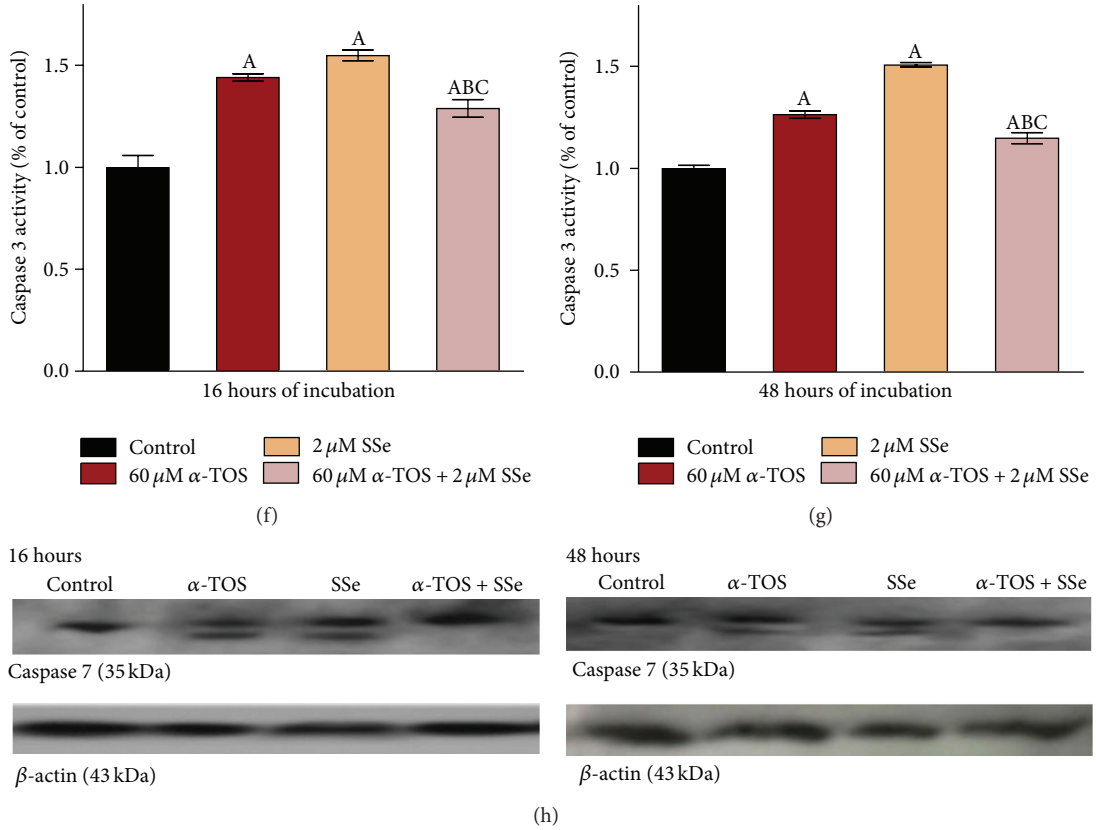
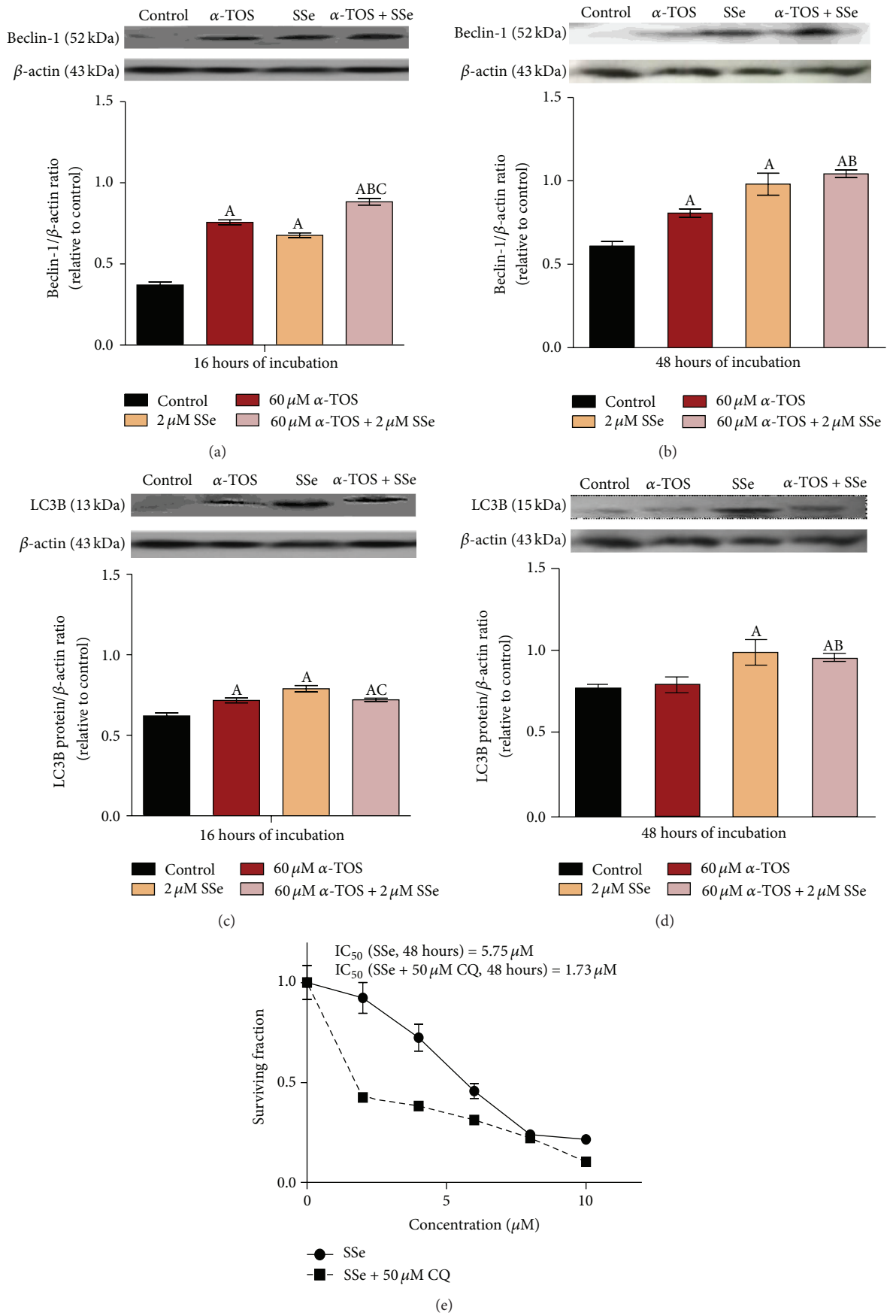


Figure 4: Effect of 60 μM α-TOS, 2 μM SSe, and their combination on Bcl-2 protein levels after (a) 16 and (b) 48 hours, on Mcl-1 protein levels after (c) 16 and (d) 48 hours, on (e) caspase 9 and (h) caspase 7 activation after 16 and 48 hours using western blot technique, and on enzymatic activity of the caspase 3 class of proteases in cell lysate of MCF7 cells after (f) 16 and (g) 48 hours of exposure using ELISA technique. Western blot results were expressed as means ± SD of 3 independent experiments ($n = 3$). ELISA results are expressed as means ± SD of 2 replicate experiments ($n = 4$). Statistical significance of results was analyzed by one-way ANOVA using Tukey's multiple comparison test. "A" significantly different from the respective control at $P < 0.05$; "B" significantly different from respective α-TOS at $P < 0.05$; "C" significantly different from respective SSe at $P < 0.05$.

death. It was suggested that the resultant activation of the caspases cascade by SSe may be ROS independent and an alternative pathway might be considered [38, 42, 44]. In addition, Park et al. [44] found that treatment of human lung carcinoma cell line with SSe resulted in an early modulation of the extrinsic apoptotic pathway, represented by an upregulation in the expression of Fas and death receptor, which was coupled with decreased expression of pro-Bid suggesting that truncated Bid might have served to connect both extrinsic and intrinsic apoptotic pathways. This was confirmed by suppression in the expression of procaspases 8, 9, and 3 by SSe, confirming activation of caspases by SSe via both apoptotic pathways. On the other hand, SSe was declared by others as a prooxidant catalyst [45–47]. Moreover, in this study SSe also activated autophagy by an increased expression of protein levels and presence of AVOs in the cytoplasm of SSe-treated cells. Available evidence suggests that SSe may stimulate or inhibit autophagy by diverse mechanisms including superoxide-targeting mitochondria [48] and mTOR signaling [49] or via beclin-1 transcriptional inhibition linked to heat shock protein 90 and nuclear factor kappa B [50]. The outcome of the combined effect of α-TOS with SSe in this study was found to be antagonistic with decrease in caspases activation compared to α-TOS alone. SSe did not affect the cellular uptake of α-TOS from the culture medium excluding that the decrease in cytotoxicity may be due to decrease in drug concentration. The antagonistic effect of SSe may contribute to inhibition of ROS induced by α-TOS and increase of antioxidant (glutathione) level by SSe which counteract the oxidative stress induced by α-TOS. It was declared that antioxidants could partially inhibit α-TOS-induced cell death in ROS dependent cells and that caspase-dependent apoptotic pathway is always involved in α-TOS-induced cell death, regardless of ROS dependence of cells. Moreover, it was found that inhibition of ROS generation did not inhibit the activation of caspases and that another mediator that activates caspase-dependent apoptosis may be present without relation to ROS generation in response to α-TOS [33]. In this study, although SSe inhibited ROS levels and apoptosis in the combination regimen, the induction of autophagy was sustained which maybe contributed to the effect of SSe. Available evidence suggests that SSe may stimulate or inhibit autophagy by diverse mechanisms [49]. In addition evidence shows that autophagy in SSe-treated



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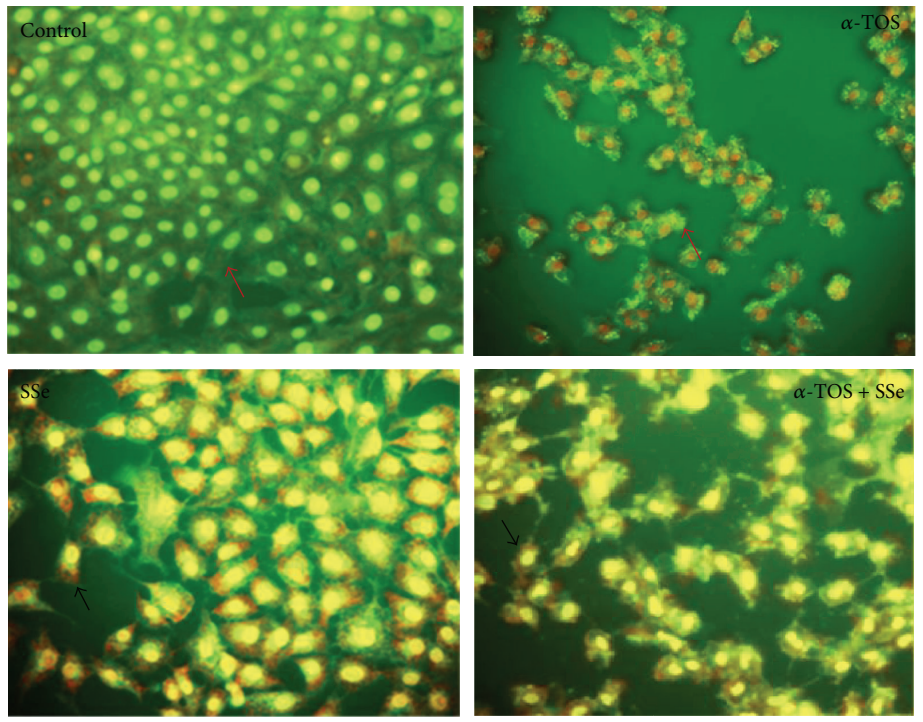

(f)

Figure 5: Effect of 60 μM α-TOS, 2 μM SSe, and their combination on beclin-1 protein level following (a) 16 hours and (b) 48 hours and on LC3B protein level following (c) 16 hours and (d) 48 hours of exposure in MCF7 cells using western blot technique and (e) effect of CQ (50 μM) on IC_{50} of SSe after 48 hours. Acridine orange stained MCF7 cells after exposure to 60 μM α-TOS, 2 μM SSe, and their combination after (f) 48 hours [original magnification (objective lens (20x))]. Cells with acidic vesicular organelles (AVOs) can be visualized by their orange fluorescence (pointed by black arrows) in the cytoplasm, whereas cells without AVOs are marked by red arrows. Western blot results are expressed as means ± SD of 3 independent experiments ($n = 3$). Statistical significance of results was analyzed by one-way ANOVA using Tukey's multiple comparison test. "A" significantly different from the respective control at $P < 0.05$; "B" significantly different from respective α-TOS at $P < 0.05$; "C" significantly different from respective SSe at $P < 0.05$.

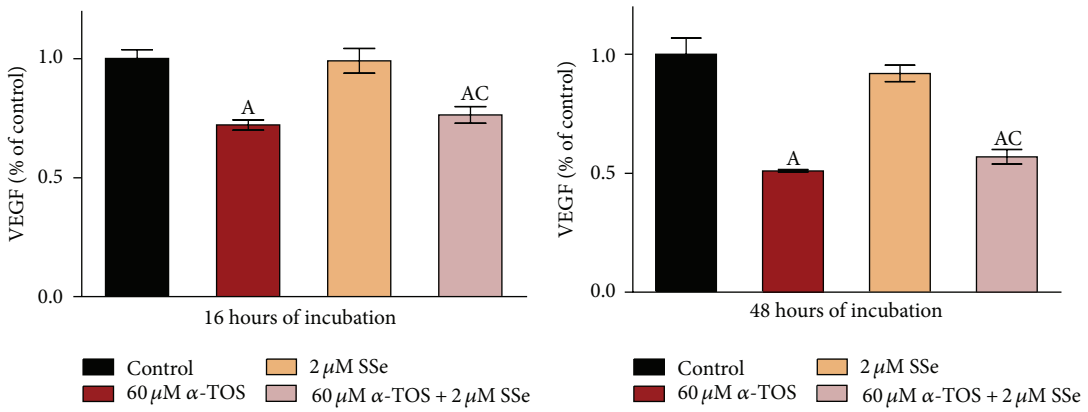

Figure 6: Combined effect of 60 μM α-TOS and 2 μM SSe on VEGF secretion by MCF7 cells following (a) 16 hours and (b) 48 hours of incubation. Results are expressed as means ± SD of 2 replicate experiments ($n = 4$). Statistical significance of results was analyzed by one-way ANOVA using Tukey's multiple comparison test. "A" significantly different from the respective control at $P < 0.05$; "B" significantly different from respective α-TOS at $P < 0.05$; "C" significantly different from respective SSe at $P < 0.05$.

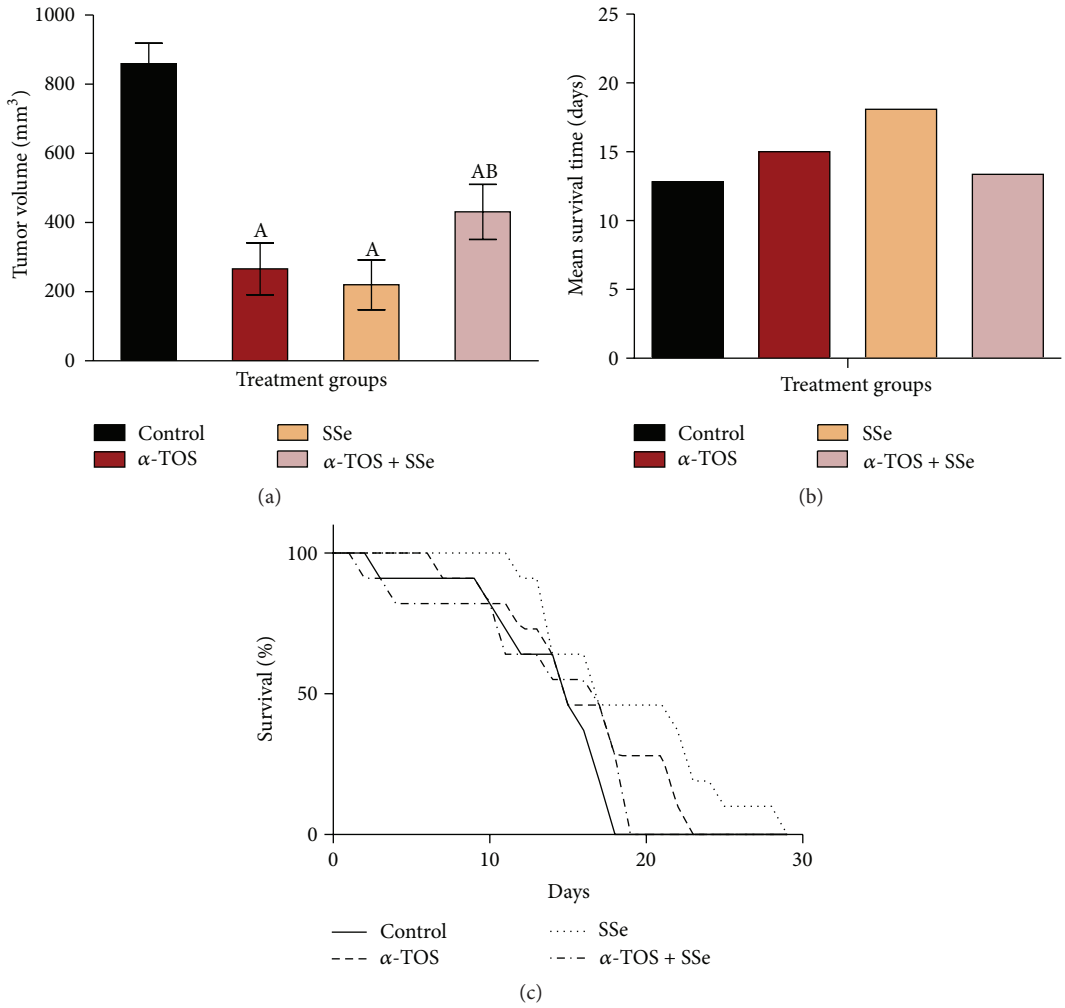

Figure 7: In vivo effects of administration of α-TOS (300 mg/kg), SSe (1 mg/kg), and their combination on (a) tumor volume of solid Ehrlich carcinoma-bearing mice, (b) mean, and (c) percent survival of EAC. Results of tumor volume are expressed as means ± SD. "A" significantly different from the respective control at $P < 0.05$; "B" significantly different from respective α-TOS at $P < 0.05$.

cells represents a concurrent process which reduces apoptosis rate [49]. To elucidate the relationship between both apoptosis and autophagy in our study, we treated cells with an autophagy inhibitor CQ. We noticed a dramatic decline in survival of cells treated with SSe and CQ was observed suggesting a prosurvival mechanism. Previous studies by Ren et al. [49], Park et al. [44], and Králová et al. [51] also confirmed a prosurvival role for SSe-induced autophagy. Although it is known that ROS can trigger autophagic cell death, other studies showed that ROS functions as a survival mechanism via induction of cytoprotective autophagy in several cancer cells [52, 53]. These types of cells induced autophagy as a means of adaption to stressful conditions.

Our in vitro findings were confirmed in vivo showing that α-TOS and SSe alone were capable of reducing the volume of solid tumor. However, the coadministration of α-TOS and SSe resulted in significantly larger tumor volume compared to α-TOS treatment group. Several reports have also documented the in vivo antitumor effects of α-TOS in lung cancer tumor [5] and colon cancer tumor [54], as well as reducing the vitality of EAC-bearing mice [55]. In addition SSe also reduced the volume of solid tumor in mice as shown by us and others [56]. In conclusion, using complementary therapies with conventional cancer therapy for symptom management and enhancement of quality of life should be intensively studied. SSe in low concentration may protect MCF7 cells from cell death induced by α-TOS via inhibiting apoptosis and induction of prosurvival autophagy.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported and encouraged by Dr. Mahmoud M. Al Marzabani who contributed to the development of the research point.

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