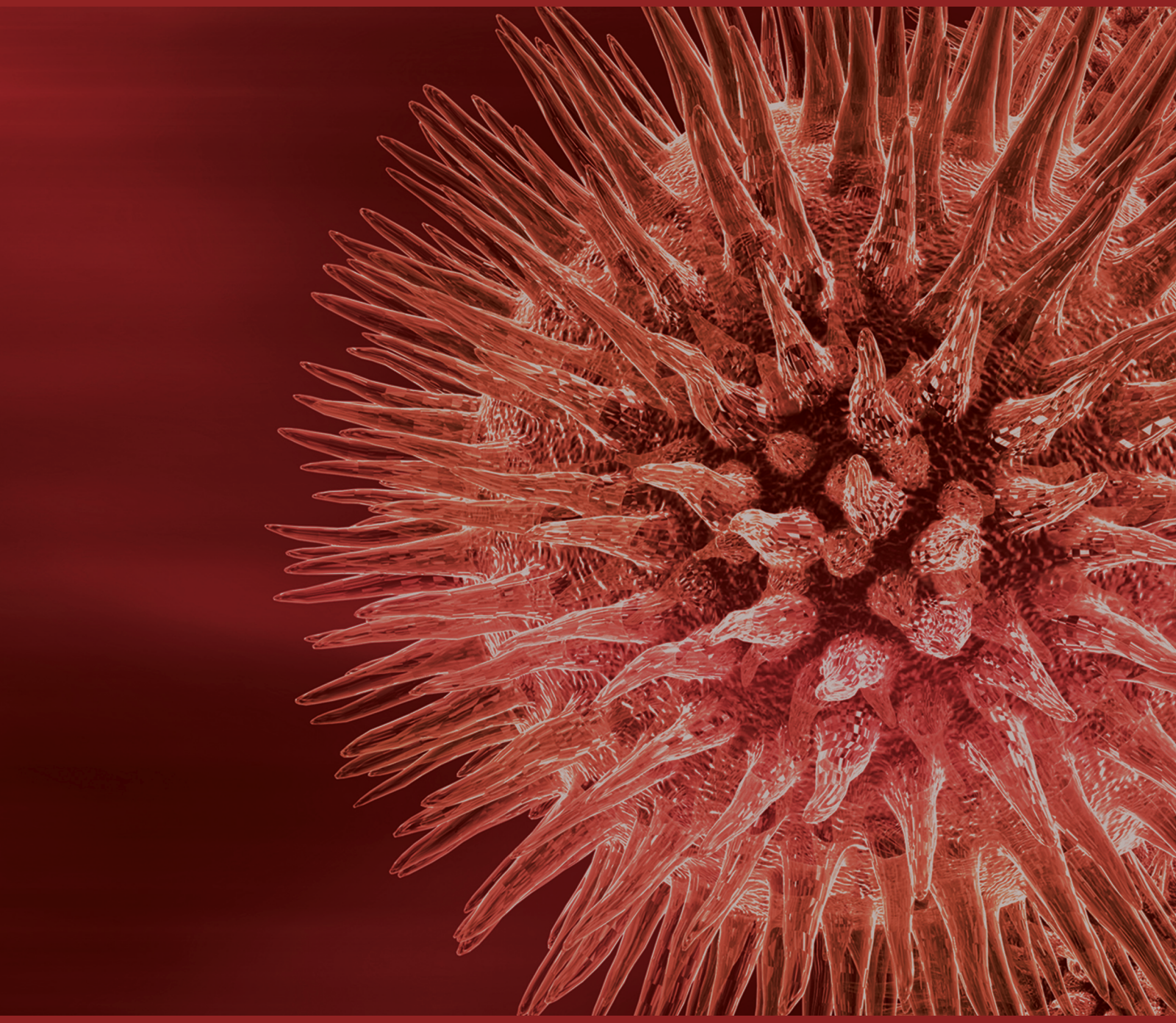
BioMed Research International

Pharmacological Application of Antiradical Compound Properties

Guest Editors: Alethéa Gatto Barschak, Francieli Moro Stefanello, Claiton Leonetti Lencina, Filippo De Simone, and Wilson João Cunico Filho



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Editorial

Pharmacological Application of Antiradical Compound Properties

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The reactive species are part of normal human metabolism and are involved in many physiological mechanisms. However, when reactive species are overproduced and antioxidant defenses are insufficient to control oxidation, an unbalance can occur, leading to cellular oxidative damage. This injury has been associated with many diseases including inflammation, cancer, cardiovascular disease, diabetes, and neurodegenerative diseases.

The population is ageing and chronic diseases affect millions of people around the world. Thus more effective treatments are necessary to provide improved quality of life for individuals. In this context, there has been growing interest in research involving natural and synthetic antioxidants which can emerge as alternative therapy and/or prevention of various chronic degenerative diseases.

In this special issue, we presented 11 original research papers and 3 review articles that describe the antiradical compound properties. The original papers explored in this special edition include a wide variety of topics such as natural products against injury (A. A. Soares et al. and S. Murthy et al.), antioxidant properties of natural products (E. Gregoris et al., A. Hashim et al., and A. Zajdel et al.), effect of glutathione on immune system (D. Morris et al.), antioxidant mechanism of buckminsterfullerene C^{60} (V. A. Chistyakov et al.), and spin labeled analogues of anticancer drugs in prevention of injury (V. Gadjeva et al.).

The review articles discuss the role of antioxidants in chronic diseases: cardioprotective effect of vitamins (R. Rodrigo et al.), antidiabetic potential of curcumin analogues (Y. Son et al.), and detrimental and protective effects of fructose (H. M. Semchyshyn).

We believe that a better understanding of action of natural and synthetic antioxidants may contribute to the development of a new therapeutic approach in many different diseases, improving the patient's quality of life.

Acknowledgments

We would like to thank the authors for their contribution and the reviewers for their collaboration.

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Francieli Moro Stefanello
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Research Article

Phytic Acid Inhibits Lipid Peroxidation *In Vitro*

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Phytic acid (PA) has been recognized as a potent antioxidant and inhibitor of iron-catalyzed hydroxyl radical formation under *in vitro* and *in vivo* conditions. Therefore, the aim of the present study was to investigate, with the use of HPLC/MS/MS, whether PA is capable of inhibiting linoleic acid autoxidation and Fe(II)/ascorbate-induced peroxidation, as well as Fe(II)/ascorbate-induced lipid peroxidation in human colonic epithelial cells. PA at 100 μM and 500 μM effectively inhibited the decay of linoleic acid, both in the absence and presence of Fe(II)/ascorbate. The observed inhibitory effect of PA on Fe(II)/ascorbate-induced lipid peroxidation was lower (10–20%) compared to that of autoxidation. PA did not change linoleic acid hydroperoxides concentration levels after 24 hours of Fe(II)/ascorbate-induced peroxidation. In the absence of Fe(II)/ascorbate, PA at 100 μM and 500 μM significantly suppressed decomposition of linoleic acid hydroperoxides. Moreover, PA at the tested nontoxic concentrations (100 μM and 500 μM) significantly decreased 4-hydroxyalkenal levels in Caco-2 cells which structurally and functionally resemble the small intestinal epithelium. It is concluded that PA inhibits linoleic acid oxidation and reduces the formation of 4-hydroxyalkenals. Acting as an antioxidant it may help to prevent intestinal diseases induced by oxygen radicals and lipid peroxidation products.

1. Introduction

Free radicals in living organisms play an important role by their beneficial and harmful effects in both physiological and pathological processes. Excessive generation of free radicals can induce lipid peroxidation and oxidative damage of other biomolecules. Lipid peroxidation leads to the formation of a number of different chain length saturated and unsaturated aldehydes and other carbonyl compounds, which contribute to peroxidative cell damage by inhibiting DNA, RNA, and protein synthesis, blocking respiration and depleting glutathione pool (see [1] for review). These compounds, especially 4-hydroxyalkenals, are sufficiently long-lived products to attack target molecules distant from the site of formation and to impair their structure/function. The two most toxic 4-hydroxyalkenals are 4-hydroxynonenal (HNE) and 4-hydroxyhexanal (HHE) [2, 3]. Uncontrolled lipid oxidation has been shown to be involved in the development of many different pathological conditions such as age-related diseases, malignancy, infective diseases, and injuries [1–3]. However, these diseases may have multifactorial origin including oxidative stress derivation. Furthermore, several environmental risk factors may influence—usually accelerate or enhance—the harmful effects of free radicals. Oxidative stress results from an imbalance between free radical formation and their elimination, so antioxidants are very important players in the battle against excessive free radical generation. Therefore, there is still an increasing interest in developing efficient antioxidants that can protect against cell injury without showing toxic effects [4, 5].

Phytic acid (PA), also known as inositol hexaphosphate, inositol hexakisphosphate, IP6, or InsP6, is the main storage form of phosphorus in plants and is especially abundant in grains, nuts, legumes, and oil seeds, where it can make up 1%–5% of the edible portion. This compound demonstrates various biological activities such as antioxidant [6], anticarcinogenic [7–9], and hypoglycemic or hypolipidemic [10, 11]. The lower inositol phosphates, such as IP4 and IP3, may play roles in mediating cellular responses and have been noted as having a function in second messenger transduction systems. Dietary PA possesses ability to bind minerals, toxic trace elements, proteins and, thus, to influence their solubility, absorption, and digestibility. PA complexes with these

elements are insoluble at physiological pH, and consequently, they exhibit low bioavailability. For several years PA had been considered as an antinutrient, but recent papers have shown the opposite [9–12]. Regarding its relatively high binding affinity towards minerals, especially to iron, PA has been recognized as a potent antioxidant and inhibitor of iron-catalyzed hydroxyl radical formation via the Fenton-type reaction [6, 12, 13]. Although the antioxidant capacity of PA has been documented in food processing, there is a lack of data on its effect on the formation of hydroperoxides as primary products and aldehydes as secondary products of lipid peroxidation.

The aim of the present study was to investigate whether PA is capable of inhibiting linoleic acid autoxidation and Fe(II)/ascorbate-induced peroxidation, as well as Fe(II)/ascorbate-induced lipid peroxidation in cultured Caco-2 cells. The Caco-2 cells derived from a human colon adenocarcinoma are commonly used as *in vitro* model because they spontaneously differentiate to form confluent monolayer of polarized cells structurally and functionally resembling the small intestinal epithelium. To avoid data misinterpretation resulting from the use of nonspecific analytical methods, the amounts of particular lipid hydroperoxides and hydroxyalkenals formed during lipid peroxidation in the presence of PA were measured by HPLC/DAD and HPLC/MS/MS.

2. Material and Methods

2.1. DPPH Radical Scavenging Activity. PA (phytic acid sodium salt hydrate from rice) was obtained from Sigma Chemical Co. Free radical scavenging activity of PA was determined with the use of 1,1-diphenyl-2-picrylhydrazil (DPPH; Sigma Chemical Co.) following the methodology described by Gülçin et al. [14], wherein the bleaching rate of a stable free radical DPPH was monitored at 517 nm. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in ethanol was prepared and 1 mL of this solution was added to 3 mL of PA solution in water at different concentrations (1–500 μM). After 30 minutes, the absorbance was measured at 517 nm. The capability of scavenging the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging effect (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (1)$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance measured in the presence of PA.

2.2. Ferrous Ions Chelating Activity. The chelation of ferrous ions by PA was estimated by the method of Dinis et al. [15]. PA at various concentrations (1–500 μM) in water (0.4 mL) was added to the solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL, Sigma Chemical Co.), and the total volume was adjusted to 4 mL with ethanol. The mixture was shaken vigorously and left at room temperature for 10 minutes. Absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe(II) complex formation was calculated using the following formula:

$$\text{Metal chelating effect (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (2)$$

where A_0 is the absorbance of control and A_1 is the absorbance measured in the presence of PA. The control contained $FeCl_2$ and ferrozine.

2.3. Measurement of Linoleic Acid Oxidation

2.3.1. Linoleic Acid Oxidation. Linoleic acid (Sigma Chemical Co.) micelles were used as model lipid system to determine the effect of PA on autoxidation or induced lipid peroxidation. The reaction mixtures contained linoleic acid (1 mM) and dispersed in HEPES buffer (50 mM, pH 7.4; Sigma Chemical Co.) and PA at various concentrations (1–500 μM). Linoleic acid peroxidation was induced by adding ferrous ions ($FeCl_2$, Sigma Chemical Co.) and ascorbic acid (Sigma Chemical Co.) to the final concentrations of 20 μM and 100 μM, respectively. Incubations were carried out for 24 hours at 37°C in a water bath with gently shaking. After incubation, each sample was diluted with methanol (1 : 1; vol/vol), filtered (filter Millex GV13, pore diameter 0.22 μm; Millipore), and immediately analyzed by HPLC.

2.3.2. HPLC Analysis of Linoleic Acid and Its Hydroperoxides. Analytical reverse-phase HPLC was performed with a Hewlett-Packard model 1050 liquid chromatograph equipped with a HP 1100 diode array detector and interfaced to HPLC ChemStation A.06.03 (HP). The incubation products were separated on the Eurospher 100 C18 column (particle size 5 μm, 250 × 4 mm; Saulentechnik Knauer) eluted isocratically with acetonitrile : water : phosphoric acid (80 : 20 : 0.1) at a flow rate of 1 mL/min at 35°C. The eluent was monitored at 234 nm for linoleic acid hydroperoxides formation and at 206 nm for linoleic acid disappearance. These compounds were identified by matching their retention times and UV spectra with authentic standards. Quantification was made based on the calibration curves.

2.4. Measurement of Secondary Products of Lipid Peroxidation in Caco-2 Cells

2.4.1. Cell Culture. Caco-2 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were grown in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES (Gibco). The cell cultures were maintained at 37°C in a 5% CO_2 atmosphere.

PA at nontoxic concentrations (0.1 mM or 0.5 mM) [16] was added to the cell cultures 1 hour prior the induction of peroxidation by Fe(II) and ascorbic acid (20 μM and 100 μM, resp.). Control cells were treated with Fe(II)/ascorbic acid without PA addition and cultured for 24 hours.

2.4.2. Preparation of the Dinitrophenylhydrazone (DNP) Derivatives of 4-Hydroxyalkenals. DNP derivatives of the

standard aldehydes were prepared according to the commonly used method described by Esterbauer et al. [17]. After 24 hours in culture, the cells were washed twice with PBS, harvested by scraping, centrifuged (10 000 g, 10 min), and immediately frozen in liquid nitrogen. The frozen cells (40–60 mg) were mixed with 1 mL of methanol, vortexed (10 min) for extraction, and again centrifuged (10 000 g, 10 min). The extraction was repeated twice. The supernatants were combined and added to the equal volumes of the DNPH reagent. The derivatization was carried out for 1 hour in dark at room temperature. The DNPH reagent was freshly prepared by dissolving 35 mg of 2,4-dinitrophenylhydrazine (Sigma Chemical Co.) in 100 mL of 1 M HCl and extracted twice with 50 mL hexane for removal of impurities. The obtained derivatives after a subsequent centrifugation (10 000 g, 15 min) were dissolved in 0.3 mL of acetonitrile and filtered (0.22 μm, Millipore). This procedure was adapted from a similar method proposed for malondialdehyde (MDA) and HNE derivatization by Deighton et al. [18].

2.4.3. Liquid Chromatography/Mass Spectrometry Determination. The 20 μL samples containing DNP-derivatives were injected on a C-18 column (Saulentechnik Knauer; 250 × 2 mm, 5 μm) and separated using a gradient mixture of water, methanol, and acetonitrile acidified with formic acid on the HPLC system HP 1100 with a diode array dete ctor from which the effluent was directed to the API 2000 PE-Sciex mass spectrometer. The following gradient pro gram was used for elution: 0 min – methanol : acetonitr ile : water : formic acid (20 : 30 : 50 : 0.1), 10 min – metha nol : acetonitrile : water : formic acid (20 : 60 : 20 : 0.1), 14 min – methanol : acetonitrile : water : formic acid (20 : 80 : 0 : 0.1). The other chromatographic parameters were as follows: flow rate 0.4 mL/min, temperature 35°C, run time 35 min, post time 12 min, and DAD detection range 200–400 nm.

The mass spectrometer equipped with atmospheric pressure ionization source worked in the negative ionization mode. The ion source and detector parameters were identical in each run (CUR 50; IS −3800 V; TEM 350; GS1 60; GS2 85; CAD 2; CE 18 eV; DP −25; FP −200; EP 10). Detection was performed in multiple reaction monitoring (MRM) mode, using characteristic mass pairs specific for each analyzed compound (m/z 293 → 167 for 4-hydroxyhexanal, m/z 335 → 167 for 4-hydroxynonenal). The concentrations of HHE and HNE were related to the amount of total cell protein determined by the standard Bradford method.

2.5. Statistical Analysis. The data obtained from 3 independent series of experiments (each in triplicate) were expressed as mean values ± standard deviations. Statistical significance analysis was based on analysis of variance (ANOVA) followed by Tukey's HSD test. The P value of less than 0.05 was considered significant. Statistical analysis was performed using Statistica 10 PL software for Windows (StatSoft, Poland).

3. Results and Discussion

A number of methods have been developed to determine the antioxidant capacities of chemical compounds and to evaluate different antioxidant mechanisms [19]. The study presented in this paper has focused on the measurement of inhibition of linoleic acid autoxidation and catalytic oxidation and Fe(II)/ascorbate-induced lipid peroxidation in a human colon Caco-2 cells by PA with the use of the HPLC/MS/MS method.

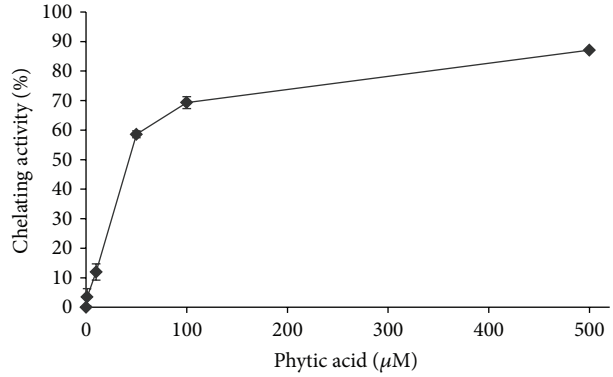

Figure 1: Ferrous ions chelating effect of phytic acid at various concentrations (0–500 μM) measured by ferrozine/$FeCl_2$. Results are the mean ± SD of 3 experiments.

In the present study, the antioxidant properties of PA at its various concentrations (1–500 μM) were examined by using the method based on DPPH scavenging and ferrous ions chelating ability determination. PA in the above concentration range did not scavenge DPPH radical ($P > 0.05$). These results are in agreement with earlier findings, where the scavenging effect of PA was observed after its irradiation only and it was positively correlated with irradiation dose [20, 21]. Ahn et al. [22] conducted a similar study to evaluate antioxidant activities of irradiated PA and ascorbic acid. Irradiated PA showed a significantly higher DPPH radical scavenging activity than ascorbic acid at the same concentration (800 μM).

One of the most commonly used systems for testing the chelating activity on Fe(II) is ferrozine/$FeCl_2$. The PA chelating activity in this system is presented in Figure 1. The observed effects were concentration dependent. PA exhibited 11.9, 58.6, 69.3, and 87.1% of ferrous ions chelation at 10, 50, 100, and 500 μM, respectively. These results confirm the findings that PA is an antioxidant acting as a potent inhibitor of iron-catalyzed radical formation by chelating free iron and blocking its coordination sites. Sakač et al. [23] observed the inhibition of hydroxyl radical generation over a wide range of phytate/iron ratio (1 : 5.5–1 : 22) and found that one phytate molecule could bind up to 6 divalent cations. At the concentration of 117 μM, PA exhibited chelating activity of 96% in ferrozine/$FeSO_4$ system [23]. It was demonstrated with the use of BPS (bathophenanthroline) based method that both standard PA and purified PA obtained from rice bran, exhibited an iron chelating capacity, which was dependent on its concentration and contact time with iron before adding BPS [24]. Ahn et al. [22] confirmed the antioxidant activity of PA by the ferric reducing antioxidant power (FRAP) test.

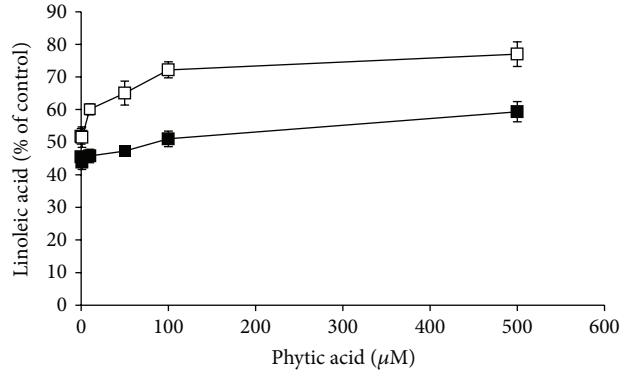

Figure 2: Effect of phytic acid (0–500 μM) on the 24-hour lasting autoxidation (□) and Fe(II)/ascorbate-induced peroxidation (■) of linoleic acid expressed as percent of the control. Results are the mean ± SD of 5 experiments.

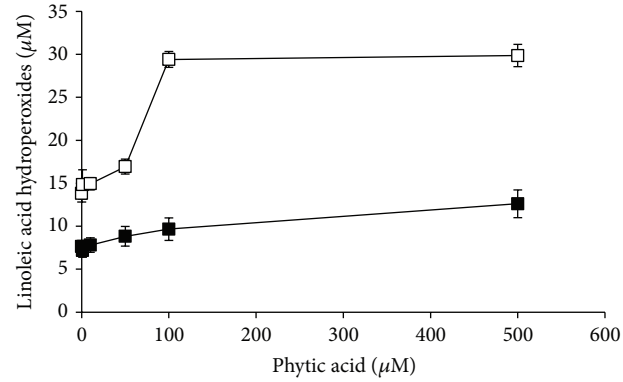

Figure 3: Effect of phytic acid (0–500 μM) on linoleic acid hydroperoxide concentration (μM) after 24-hour lasting autoxidation (□) and Fe(II)/ascorbate induced linoleic acid peroxidation (■). Results are the mean ± SD of 5 experiments.

Transition metal ions are involved in lipid peroxidation by decomposing lipid peroxides into their peroxyl and alkoxyl radicals. These radicals abstract hydrogen atom from polyunsaturated fatty acid molecule and perpetuate a chain reaction of lipid peroxidation [1]. PA has antioxidant functions by virtue of forming a unique iron chelate, and it suppresses iron-catalyzed oxidative reactions [6]. Consequently, such actions of PA may be involved in its inhibitory effect on lipid oxidation [13]. Therefore, in the present study it was investigated whether PA was able to inhibit autoxidation and Fe(II)/ascorbate-induced peroxidation of linoleic acid. In this analysis, the oxidant Fe(II)/ascorbate pair was used because iron can be reduced by ascorbate and, thus, cause a significant increase in the formation of reactive oxygen species and the extent of lipid peroxidation [25]. As shown in Figure 2, PA at 100 μM and 500 μM effectively inhibited the decay of linoleic acid, both in the absence and presence of Fe(II)/ascorbate ($P < 0.05$). The observed inhibitory effect of PA on Fe(II)/ascorbate-induced lipid peroxidation was lower (10–20%) compared to that of autoxidation, probably due to its direct interaction with Fe(II) ions. PA did not change linoleic acid hydroperoxides concentration levels after 24 hours of Fe(II)/ascorbate-induced peroxidation ($P > 0.05$), and only about 3% of linoleic acid was converted into hydroperoxides (Figure 3). In the absence of Fe(II)/ascorbate, PA at 100 μM and 500 μM significantly suppressed decomposition of linoleic acid hydroperoxides ($P < 0.05$; Figure 3). Their concentrations increased about twofold. With the use of 100 μM and 500 μM PA, about 10% and 13% of linoleic acid were converted into hydroperoxides. These results are in agreement with the findings of Graf et al. [13], who demonstrated that phytate prevented peroxidation of arachidonic acid driven by ascorbic acid and iron by shifting the redox potential of iron Fe(II) → Fe(III). Fe(II) stimulates production of lipid oxyradicals, whereas Fe(III) is relatively inert. Studies using liposomal membranes demonstrated that PA derived hydrolytic products containing three or more phosphate groups were able to inhibit iron induced lipid peroxidation, although their effectiveness decreased with dephosphorylation degree. The products of PA hydrolysis also prevented iron induced decomposition of phosphatidylcholine hydroperoxide [26]. Ahn et al. [20] reported that the antioxidant activity of PA was slightly increased by irradiation in an aqueous lipid model system; although at higher concentrations (400 μg/mL), this activity remained the same compared to that of nonirradiated PA or it was even reduced.

Antioxidants may inhibit decomposition of lipid hydroperoxides by acting as radical scavengers, metal chelators, or reducers of hydroperoxides to more stable hydroxyl compounds [1]. These effects of PA are mainly associated with its chelating activity. The results of the present study suggest that PA at higher concentration (100 μM, 500 μM) scavenged the reactive oxygen species produced during autoxidation of linoleic acid and prevented decomposition of lipid hydroperoxides. On the other hand, the earlier report postulated, that PA did not act as the chain-breaking antioxidant capable of scavenging free radicals and thus preventing lipid oxidation. This can be explained by the structural feature of PA which is a lack of a hydrogen atom to be transferred to peroxyl radicals [22, 23]. This conclusion was later confirmed by the results of AOA (β-carotene bleaching) test in β-carotene/linoleic acid model system. PA did not exhibit any antioxidant activity in the tested concentration range (3.7–58.6 μM) [23]. Sakač et al. [23] found that it did not inhibit thermal oxidation of hydroperoxide-enriched soybean oil (HESO) but influenced catalytic oxidation of HESO by chelating Fe(II) and inhibited generation of lipid oxyradicals that were detected in the form of PBN-OOL/-OL spin adducts.

The possibility of utilizing PA as an antioxidant in food processing and storage was documented by Stodolak et al. [27], who found the improvement of oxidative stability of raw and cooked meat. It is known that PA can effectively and dose-dependently inhibit lipid peroxidation in beef and pork homogenates [28, 29]. Park et al. [21] concluded that irradiated PA significantly inhibited lipid oxidation in meat homogenates compared to the control sample. Moreover, it was shown that irradiated PA was capable of preventing the loss of the heme iron and myoglobin formation during meat

storage. Sorour and Ohshima [30] confirmed the antioxidant potential of pure PA (PA sodium salt) and PA extracted from wheat bran. They measured TBARS level, oxygen absorption, and total lipid hydroperoxide content in cod liver oil o/w emulsion oxidized at 40°C under dark. PA at a concentration of 4 mM inhibited total hydroperoxides and TBARS formation by 62% and 67% of control, respectively. In this study, PA appeared to be more effective than ascorbic acid and therefore it was recommended as a food antioxidant that prolongs the stability of fish lipids or fish meats [30].

Lipid peroxidation has been described to cause gradual changes in cellular membrane structure, ultimately leading to the loss of function and integrity [1]. Moreover, aldehydic lipid peroxidation products, especially 4-hydroxyalkenals (HHE, HNE), due to their high reactivity, show marked biological effects. Experimental and clinical evidence suggest that 4-hydroxyalkenals can act as bioactive molecules under physiological and/or pathological conditions. These compounds can affect and modulate, even at very low-nontoxic concentrations, several cell functions, including signal transduction, gene expression, cell proliferation, and differentiation, cellular growth inhibition, or apoptosis [2, 3]. Numerous methods have been developed to measure lipid peroxidation products and lipid peroxidation damage in tissues, cells, and body fluids. Malondialdehyde (MDA) reactivity with 2-thiobarbituric acid (TBA) is the principal analytical method for evaluating lipid peroxidation [31]. Nevertheless, the specificity of these methods can be questioned because aldehydes other than MDA can react with TBA. Moreover, TBA assay conditions such as high temperature and low pH may themselves cause the oxidation of lipids. Therefore, several analytical methods have been proposed using chromatographic techniques coupled with sensitive detectors for determination of aldehydic products in biological systems [32, 33]. In the present study, the HPLC/MS/MS method was applied for the quantitative analysis of aldehydic lipid peroxidation products formed in Caco-2 cells. PA at the tested nontoxic concentrations (100 μM and 500 μM) significantly decreased HHE and HNE levels (about twofold) in Caco-2 cells (Table 1). The obtained results correspond with the findings that dietary intrinsic phytate from corn and soy was protective against lipid peroxidation in the colon of pigs [34], rats [35], and mice [36] supplemented with a moderately high level of dietary iron. Another experiment performed on high fat-fed mice receiving rice bran or pure PA in the diet showed that such supplementation suppressed lipid peroxidation as evidenced by the significantly lower TBARS levels in plasma and erythrocytes [10]. Biological activity of PA *in vivo* in most cases seems to be associated with its antioxidant activity, chelation of Fe(III), and suppression of hydroxyl radical formation. Although this mechanism is widely recognized data on antioxidant effect of PA *in vivo* published in the recent years are very limited.

Table 1: Effect of phytic acid on aldehydic lipid peroxidation product levels in Caco-2 cells after 24-hour lasting Fe(II)/ascorbate-induced lipid peroxidation (mean ± SD of 4 experiments).

Phytic acid [mM]	4-Hydroxyhexenal [nmol/g protein]	4-Hydroxynonenal [nmol/g protein]
0	8.24 ± 1.69	0.44 ± 0.09
0.1	3.89 ± 0.93*	0.24 ± 0.07*
0.5	2.83 ± 0.89*	0.18 ± 0.06*

*Statistically significant difference in comparison with control; $P < 0.05$.

4. Conclusion

PA can scavenge the reactive oxygen species produced during autoxidation of linoleic acid and reduce the formation of 4-hydroxyalkenals. It can act as a natural antioxidant and prevent intestinal diseases induced by oxygen radicals and lipid peroxidation products.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Possible Mechanisms of Fullerene C_{60} Antioxidant Action

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Novel mechanism of antioxidant activity of buckminsterfullerene C_{60} based on protons absorbing and mild uncoupling of mitochondrial respiration and phosphorylation was postulated. In the present study we confirm this hypothesis using computer modeling based on Density Functional Theory. Fullerene's geroprotective activity is sufficiently higher than those of the most powerful reactive oxygen species scavengers. We propose here that C_{60} has an ability to acquire positive charge by absorbing inside several protons and this complex could penetrate into mitochondria. Such a process allows for mild uncoupling of respiration and phosphorylation. This, in turn, leads to the decrease in ROS production.

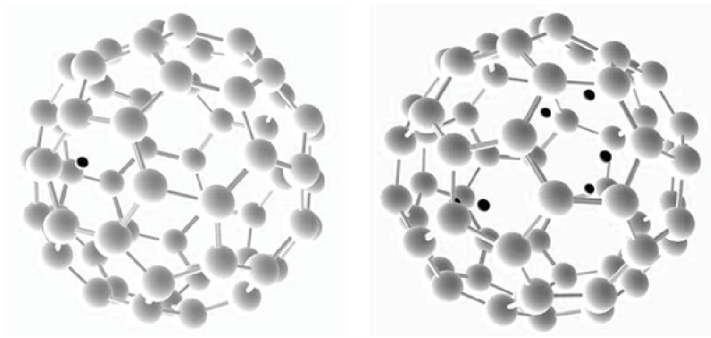
1. Introduction

Reactive oxygen species (ROS) are able to cause oxidative damage to DNA, lipids, and proteins and are known to be the key regulators of cellular signaling. In spite of the criticism from a number of researchers [1] free-radical theory occupies a pivotal position in modern biological concepts of aging [2]. The ability to retard senescence is typical for many antioxidants [3–5]. Well-known ability of fresh vegetables, fruits, red wines, and spices to stimulate longevity is largely determined by the existence of compounds such as deprotonated xanthones [6], carotenoids [7], anthocyanins and pyranoanthocyanins [8], and flavonoids and terpenoids [9]. These compounds exhibit a broad spectrum of oxyradical quenching activity based on reactions of single electron transfer, hydrogen atom transfer, sequential electron proton transfer, proton coupled electron transfer, radical adduct formation, and iron chelation [5, 9–13].

In the recent study Baati et al. [14] showed that the oral administration of the fullerene C_{60} suspension in olive oil retards senescence of rats. Herewith, median and maximum life span increase approximately twice. Moreover, it was shown that rats treated with fullerene C_{60} demonstrated high resistance to carbon tetrachloride. Toxicity of this substance is mediated by ROS generation [15]. According to this fact and results of biochemical tests fullerene C_{60} was proposed to be of high antioxidant activity *in vivo*. Due to the free-radical theory of aging, highly active antioxidant activity can be the basis for unique antiaging (geroprotective) properties.

Fullerene C_{60} is known to be able to inactivate hydroxyl radicals by attaching to double bonds [16]. However, this mechanism cannot explain sufficient (near two times) increase in lifespan of rats. Such kind of antioxidative activity is also attributed to natural phenolic antioxidants that do not possess high senescence retarding activity [17]. We propose that there is an additional mechanism involved in fullerene anti-aging activity. Respiratory chain located in the inner mitochondrial membrane is the main source of superoxide anion radicals, which lead to a cascade of other toxic ROS. In this connection mitochondrial-targeted antioxidants like lipophilic cations (Skulachev ions) with antioxidant load [18] are the most effective antiaging agents (geroprotectors) among synthetic compounds.

Accumulation of Skulachev ions in the mitochondria is based on the transmembrane potential difference generated as a result of electron transport chain activity. The outer side of inner membrane of mitochondria has positive charge and the inner side has negative charge. So, lipophilic cations are concentrating in mitochondria via electric field forces [18].



(a) (b)

Figure 1: The results of DFT geometry optimization for one (a) and six (b) protons and fullerene. Initially protons were placed outside the fullerene and then the configuration that has the minimum value of total energy was found as a result of DFT geometry optimization. As a result, all protons appeared to be inside the fullerene. For the simulation, GGA-BLYP exchange-correlation potential was used. Carbon atoms are shown in grey and protons are shown in black.

The lipophilic properties of fullerene C_{60} are well known [19]. In addition, Wong-Ekkabut et al. showed using molecular dynamics simulations [20] that C_{60} fullerene is capable of penetrating into membrane and accumulates in the middle of lipid bilayer. However, the simulation does not consider the possible presence of fullerene and/or membrane charge. We suppose that fullerene is capable of absorbing protons and obtaining positive charge, which allows it to be delivered into the mitochondria. Thus, superoxide anion-radical generation is decreased by mild uncoupling of respiration and phosphorylation [21]. In the present study we perform theoretical analysis of the fullerene C_{60} ability to acquire positive charge and to absorb protons to prove that the proposed mechanism indeed may take place.

2. Methods

All the computer simulations were performed within the framework of Density Functional Theory (DFT) for solving Schrödinger equation [22], which has been used for the investigation of antioxidants previously [23]. In the present work, DFT implemented in ADF 2012 code was used [24]. Initially from one up to seven protons were placed outside the fullerene and then the most probable atomic configuration was found by minimizing the total energy of the system during the process of geometry optimization, that is, finding a stable configuration of the system that corresponds to the minimum of total energy. For the exchange-correlation part of molecule potential General Gradient Approximation (GGA) was used in both GGA-BLYP [25] and GGA-BLYP-D3 [25, 26] forms, but all final results were obtained using GGA-BLYP potential. Basis sets are DZ (double-ζ) within the calculations including water molecules around C_{60} and TZP (triple-ζ) within the calculations without taking into account the water molecules around "C_{60} plus-protons" system.

3. Results

At first step an interaction between single proton and fullerene was simulated. The proton was placed outside the C_{60} above one of the pentagons at the distance about 1 Å from the pentagon plane. As a result, the proton transfers into the fullerene and finally appeared to be inside the fullerene at a distance about 1.1 Å from the nearest carbon atom (Figure 1(a)). Next, more protons were added to this system; some of them were initially placed above pentagons, but most were placed above hexagons. The first two protons were placed at maximum possible distance from each other. All others were equally distributed around the fullerene. In all cases protons were "absorbed" by the fullerene, and it was so until the seventh proton was added to the system—it repulsed from the fullerene. So, the maximum amount of protons inside the fullerene consists of six protons (Figure 1(b)).

It is crucial to know the distribution of charge over C_{60} for each configuration of protons. Figure 2 shows the distribution for two, four, and six protons inside the fullerene. It can be seen that when there are two protons inside the surface of the fullerene has almost no charge. When four to six protons are added the fullerene surface obtains positive charge.

Table 1 provides information about binding energies and VDD charges [27] for each proton added to the system. Both charges on protons and relatively big C-H distances allow us to suppose that protons interact with fullerene according to donor-acceptor mechanism and do not form strong chemical bonds.

It is important to know whether the presence of other molecules near fullerene will impact the ability of protons to penetrate into fullerene or not. For this purpose we performed a simulation involving water molecules which are the most common in organisms. Though it is known that in the presence of both protons and water hydronium ions will appear, water molecules can be chosen. An exchange of protons between hydronium ions takes place in such environment, so for some small period of time protons are free.

The simulation was carried out for a fullerene with single proton placed above a pentagon and 47 water molecules randomly distributed around the fullerene. It was shown that solvent molecules do not influence the capability of a fullerene to absorb the proton.

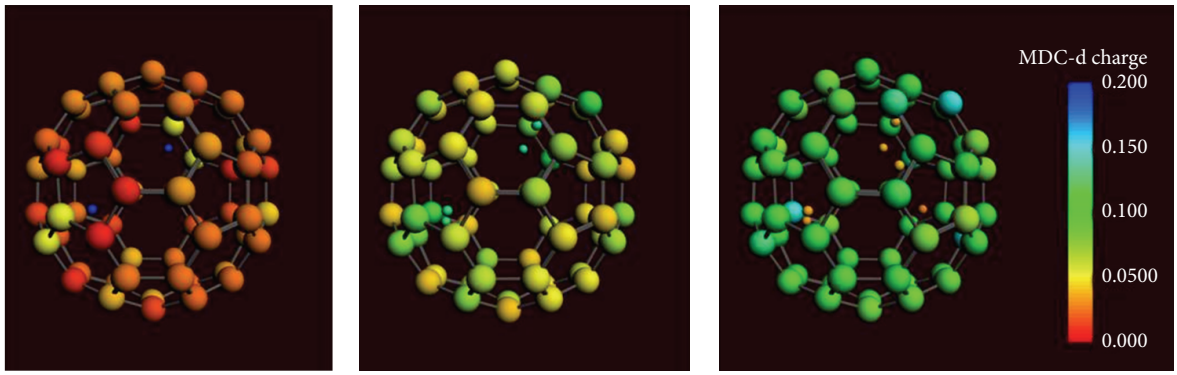


Figure 2: The distribution of charge for two, four, and six protons inside the fullerene. The charge of fullerene with two protons inside is about zero (red color) while fullerenes that have four or six protons inside obtain positive charge (green and blue color). Protons lose their positive charge starting from positive charge (blue color) to almost zero (orange color).

Table 1: Binding energies and VDD charges for different amounts of protons added to fullerene.

Number of protons	Binding energy values, eV	The Voronoi Deformation Density (VDD)
1	18.01	0.350
2	22.34	0.345 0.333
3	22.97	0.334 0.314 0.312
4	28.69	0.319 0.308 0.304 0.268
5	28.71	0.281 0.299 0.302 0.278 0.328

4. Discussion

According to our model fullerene C_{60} accumulating in mitochondria provides high radical scavenging activity in this subcellular compartment, called by Skulachev the "dirtiest place in the cell" [28]. Another effective antioxidant mechanism is based on mild uncoupling of respiration and phosphorylation. Respiratory chain obtains electrons from NADH and succinate. They are used for harmless four-electron reduction of oxygen. But the transfer of one or two electrons could produce the radicals that are dangerous to cells (such as superoxide or peroxide anions).

The specific feature attributable to the generation of ROS by mitochondria is related to the fact that the higher is the membrane potential (the larger is the difference in the concentration of protons inside and outside the mitochondria), the higher is the level of the superoxide anion production. As it was shown [29], there is steep dependence of mitochondrial superoxide-anion-radical generation on transmembrane potential ($\Delta\psi$). Even a small (10–15%) decline of $\Delta\psi$ resulted in tenfold lowering of ROS production rate.

Therefore, the so-called mild uncouplers of oxidative phosphorylation are the substances which can move some of the protons inside the mitochondria and can possess an excellent oxygen-protective effect, although they are not antioxidants in terms of chemistry [19].

DFT simulations allowed us to propose the following mechanism. C_{60} fullerene molecules enter the space between inner and outer membranes of mitochondria, where the excess of protons has been formed by diffusion. In this compartment fullerenes are loaded with protons and acquire positive charge distributed over their surface. Such "charge-loaded" particles can be transferred through the inner membrane of the mitochondria due to the potential difference generated by the inner membrane, using electrochemical mechanism described in detail by Skulachev et al. [18, 24]. In this case the transmembrane potential is reduced, which in turn significantly reduces the intensity of superoxide anion-radical production.

5. Conclusion

The proposed ability of C_{60} fullerenes to acquire positive charge allows ascribing them to the mitochondrial-targeted compounds. The key role of mitochondria in the cellular regulation makes such "charge-loaded" fullerenes be of great interest along the route for novel classes of drugs development.

Authors' Contribution

V. A. Chistyakov and Yu. O. Smirnova contributed equally to this work.

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Review Article

Antidiabetic Potential of the Heme Oxygenase-1 Inducer Curcumin Analogues

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Although there is a therapeutic treatment to combat diabetes, the identification of agents that may deal with its more serious aspects is an important medical field for research. Diabetes, which contributes to the risk of cardiovascular disease, is associated with a low-grade chronic inflammation (inflammatory stress), oxidative stress, and endoplasmic reticulum (ER) stress. Because the integration of these stresses is critical to the pathogenesis of diabetes, agents and cellular molecules that can modulate these stress responses are emerging as potential targets for intervention and treatment of diabetic diseases. It has been recognized that heme oxygenase-1 (HO-1) plays an important role in cellular protection. Because HO-1 can reduce oxidative stress, inflammatory stress, and ER stress, in part by exerting antioxidant, anti-inflammatory, and antiapoptotic effects, HO-1 has been suggested to play important roles in pathogenesis of diabetes. In the present review, we will explore our current understanding of the protective mechanisms of HO-1 in diabetes and present some emerging therapeutic options for HO-1 expression in treating diabetic diseases, together with the therapeutic potential of curcumin analogues that have their ability to induce HO-1 expression.

1. Introduction

A number of studies have suggested that diabetes mellitus, the hallmark of which consists of elevated plasma glucose, is consistently associated with oxidative stress, a chronic low-grade inflammation (hereafter referred as to "inflammatory stress"), and endoplasmic reticulum (ER) stress [1, 2]. Moreover, it is most likely that oxidative stress, inflammatory stress, and ER stress may interact with each other during pathogenesis of diabetes, and they, as a result, may be amplified to ultimately induce abnormal cell death. Thus, agents and/or cellular defense molecules that can modulate these stress responses are emerging as potential targets for intervention and treatment of diabetes. Numerous experimental studies have confirmed the important role of naturally occurring phytochemicals in prevention and treatment of diabetes, particularly associated with oxidative stress [3]. Of them, the nutritional antioxidant curcumin (Cur) has been highlighted [4]; therefore, we will discuss the therapeutic use of Cur, in the context of oxidative stress-related diseases, together with the underlying mechanisms of its action.

Turmeric is prepared by grinding dried rhizomes of *Curcuma longa*. Traditionally, turmeric has been used as a foodstuff and has been an important component of Indian medicine and traditional Chinese medicine [5]. Cur is one of the active components responsible for the majority of the medicinal properties of turmeric. Cur has been shown to protect against oxidative stress [4–6]. However, the mechanisms of actions involved in the antioxidant-related protective effects of Cur are not fully understood. Cur has been reported to have the capacity to directly quench reactive oxygen species (ROS) that can contribute to oxidative damage [7]. While this property of Cur is known to contribute to its overall protective effects, Cur can also have the capacity to activate or inhibit various cellular signaling pathways and

numerous additional regulatory molecules involved in cellular protection against oxidative stress [8]. Interestingly, recent studies have shown that Cur can attenuate cell death caused by oxidative stress, indirectly through induction and/or activation of antioxidant/cytoprotective enzymes, such as heme oxygenase-1 (HO-1) [9].

HO-1, a ubiquitous inducible cellular stress protein, serves a major metabolic function as the rate-limiting step in the oxidative catabolism of heme, leading to formation of equimolar amounts of biliverdin (BV), free iron, and carbon monoxide (CO); the BV formed in this reaction is rapidly converted to bilirubin (BR) by BV reductase [10]. It has become increasingly recognized that HO-1, an inducible enzyme, plays an important role in cellular protection [11]. The protective biological activities conferred by HO-1 include antioxidant, anti-inflammatory, and antiapoptotic properties [10, 11]. By virtue of such protective activities, HO-1 has been suggested to play important roles in pathogenesis of diabetic diseases [12]. In the present review, we will explore our current understanding of the protective mechanisms of HO-1 in diabetes and present some emerging therapeutic options for HO-1 expression in treating diabetic complications, together with the therapeutic potential of Cur analogues.

2. Cellular Stresses in Diabetes

A growing body of evidence suggests an early and central role of increased oxidative stress as a causal pathway linking with diabetic diseases [1–3]. Besides oxidative stress, ER stress and inflammatory stress are often present in the patients with diabetes, whereas they are also related to oxidative stress [1, 2]. Although roles for individual processes, such as oxidative stress, inflammatory stress, and ER stress, in diabetes have been recognized in scattered reports, how these processes are interrelated in bringing about diabetes has not been clear. However, these processes are ultimately integrated in the pathogenesis of diabetes.

2.1. Oxidative Stress. ROS, of which production is an unavoidable consequence of aerobic metabolism in animal cells, consist primarily of the various oxygen free radicals, including superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$), as well as the potent oxidizing molecules, including hydrogen peroxide (H_2O_2). At their high concentrations, ROS can react with many different macromolecules, thereby causing damage to, for example, DNA, proteins, and lipids [13]. ROS, therefore, play a major role in many disease processes. Despite their destructive activity, low/moderate levels of ROS are indispensable in several biochemical processes, including intracellular messaging and defense against microorganisms [14]. Thus, it is necessary for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them completely. This is supported by the fact that levels of ROS are tightly regulated by cellular antioxidant defense systems including small antioxidant molecules, such as glutathione, and ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) [15]. Thus, oxidative stress has been shown to describe a condition in which these cellular antioxidant defense mechanisms are insufficient to inactivate ROS, or excessive ROS are produced, or both. The novel concepts in our understanding of oxidative stress indicate that a perturbed redox circuitry could be strongly linked with the onset of diabetes.

Nicotinamide adenine dinucleotide phosphate (NADP) oxidase that can produce superoxide in response to an inflammatory response in phagocytotic cells or by receptor tyrosine kinase-mediated engagement in nonphagocytotic cells [16] and the mitochondrial respiratory chain mainly at complexes I and III in the mitochondria are believed to be major sites of ROS production under physiological and pathological conditions. In the mitochondria, ROS are spontaneously generated but rapidly converted to nontoxic molecules by cellular antioxidant molecules, such as SOD, catalase, and GPX. However, in a state of chronic nutrient/energy overload or continued exposure to glucose metabolites, the flux of nutrients through the mitochondrial respiratory chain can be increased, thereby enhancing ROS production, presumably because of insufficient capacity of cellular antioxidants, such as SOD, catalase, and GPX, to neutralize excessive ROS, and eventually inducing oxidative stress. ROS have been hypothesized to inhibit the cell signaling of the insulin receptor by blocking the pathway between insulin-receptor substrate 1 (IRS-1) and phosphatidylinositol-4,5-bisphosphate 3-kinase, thereby inducing insulin resistance (IR) [17]. This hypothesis has been supported by the findings demonstrating that animal models of IR and diabetes are characterized by persistently elevated ROS levels [18].

2.2. Inflammatory Stress. Inflammation is a response to eliminate the initial cause of cellular injury as well as the necrotic cells and tissues that result from the original insult. The mechanism causing inflammation during the pathogenesis of diabetic diseases is still under investigation. It is most likely that the immune sensors, such as pattern recognition receptors (PRRs) and other pathogen-sensing kinases, may participate in the development of diabetes. Pathogen-associated molecular pattern molecules (PAMPs) are derived from microorganisms and recognized by PRR-bearing cells of the innate immune system. In contrast, damage-associated molecular pattern molecules (DAMPs) are cell-derived and initiate and perpetuate immunity in response to trauma, ischemia, and tissue damage, either in the absence or presence of pathogenic infection [19]. Most PAMPs and DAMPs bind specific PRRs, such as Toll-like receptors (TLRs) [19]. It is most likely that DAMPs released from the necrotic cells and tissues resulting from their exposure to toxic molecules, such as ROS, may trigger inflammatory response. Interestingly, TLR4 has been reported to be activated by saturated free fatty acids (FAs) to generate inflammatory signals in macrophages, endothelial cells (ECs), and adipocytes, ultimately resulting in the production of proinflammatory cytokines, such as (TNF-α) and interleukin-1β (IL-1β), and ROS [20]. The bacterial endotoxin lipopolysaccharide (LPS) is a classical ligand for TLR4 in most cell types. The majority of the biological activity of LPS is contained within a moiety that is

acylated with saturated FAs, and removal of these FAs results in complete loss of its ability to activate TLR4, suggesting that there is a degree of similarity in structure among LPS and saturated free FAs. It is well established that elevated levels of proinflammatory cytokines are detected in patients with the IR-associated clinical states [21–23] and in experimental mouse models of diabetes [24].

2.3. ER Stress. The ER is a highly dynamic organelle responsible for protein folding, maturation, quality control, and trafficking. When the ER becomes stressed due to the accumulation of newly synthesized unfolded proteins, this condition has been referred to as an "ER stress," and the unfolded protein response (UPR) is activated to increase protein folding capacity and to decrease unfolded protein [25]. If these mechanisms of adaptation are insufficient to recover ER homeostasis, the UPR will induce cell death programs to eliminate the stressed cells, which may contribute to disease states. In animal cells, the UPR is mediated by at least three transmembrane proteins, including inositol-requiring enzyme 1 (IRE1), protein-kinase-RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [25, 26]. Under unstressed conditions, these transmembrane proteins are maintained in an inactive state by binding to the major ER chaperone, immunoglobulin heavy chain binding protein/glucose-regulated protein 78 (BiP), at the side of the ER lumen. During ER stress, BiP is displaced to interact with misfolded luminal proteins, resulting in the release of IRE1, PERK, and ATF6, and subsequently leading to their activation [25, 26]. PERK activation results in phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) leading to rapid reduction in the initiation of mRNA translation, and thus reducing the load of new proteins in the ER. Phosphorylation of eIF2α by PERK allows the translation of activating transcription factor 4 that can induce transcription of genes involved in amino acids synthesis and apoptosis, such as CCAAT/enhancer-binding protein homologous protein (CHOP) [25, 26]. ER calcium depletion, altered glycosylation, nutrient deprivation, oxidative stress, proinflammatory cytokine, DNA damage, or energy perturbation/fluctuations can interrupt the protein folding process and result in ER stress. A study has demonstrated protection against obesity-induced diabetes in mice by overexpression of ER chaperones, while knockdown of chaperones was diabetogenic [27]. In addition, treatment with chemical ER chaperones that alleviated obesity-induced ER stress led to improvement in insulin sensitivity [27].

3. HO-1 and Therapeutic Potentials

Although HO-1 is known initially for its role in heme catabolism, HO-1 has become increasingly recognized that HO-1 expression exerts a major role in cellular defense mechanisms [10, 11]. The protective activities conferred by HO-1 expression include its antioxidant, anti-inflammatory, and antiapoptotic properties [10, 11]. These protective effects of HO-1 are dependent on the generation of its enzymatic reaction products (i.e., CO, BV/BR). There is ample evidence that HO-1, in particular, can protect against diabetics (Figure 1) [12].

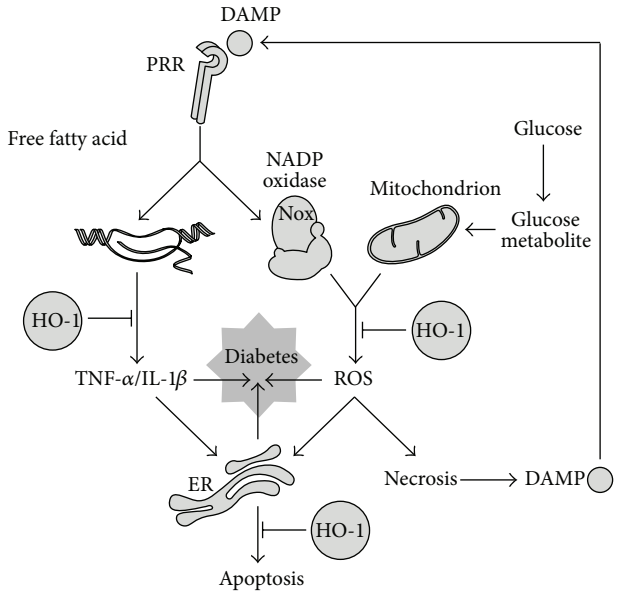

Figure 1: Therapeutic targets of HO-1 during pathogenesis of diabetes. Cells in a tissue may be exposed to oxidative stress generated mainly by mitochondria and NADP oxidase complex, inflammatory stress initiated probably by DAMP-PRR engagement, and ER stress triggered by inflammatory and oxidative stresses, and these stresses, when prolonged, may be amplified and integrated. The integration of amplified stress responses may cause one or more of diabetic complications. HO-1 expression may reduce oxidative stress, inflammatory stress, and ER stress, thereby exerting therapeutic actions.

3.1. HO-1 against Oxidative Stress. The antioxidant effect of HO-1 has been highlighted in HO-1-knockout mice [27]. As compared with wild-type mice, the liver from HO-1-knockout mice show higher levels of oxidized proteins and lipid peroxidation. Moreover, peritoneal macrophages from HO-1-knockout mice, as compared with wild-type controls, exhibit increased levels of ROS [28]. Similarly, cells from the human case of HO-1 deficiency showed increased sensitivity to oxidative injury [29]. HO-1, therefore, plays a role to counteract oxidative stress, being upregulated during oxidative stress. The specific mechanisms by which HO-1 can mediate antioxidant effect are not clear, but BV and BR, a byproduct generated during the heme catabolism, have been suggested as potential antioxidants. In fact, addition of BR to the culture medium was reported to markedly reduce the cytotoxicity produced by oxidants [10, 11]. Similarly, HO-1 expression by heme increased the resistance against oxidative cell injury; notably, this protective effect occurred only in cells that were actively producing BR [30]. It is important to note that up-regulation of HO-1 is often associated with increased ferritin [31], which sequesters redox-active iron, a toxic byproduct of heme degradation [10, 11].

3.2. HO-1 against Inflammatory Stress. The anti-inflammatory effect of HO-1 has been also highlighted in HO-1-knockout mice [27]. As compared with wild-type mice, HO-1-knockout mice exhibited hallmarks of a progressive chronic inflammatory state. Peritoneal macrophages from HO-1-knockout mice, as compared with wild-type mice, exhibited increased proinflammatory cytokines [28]. Similarly, a case of human HO-1 deficiency also exhibited hallmarks of a proinflammatory state [29]. The specific mechanisms by which HO-1 can mediate anti-inflammatory effect are not clear, but CO has been suggested as a potential mediator. Studies have shown that administration of CO inhibited the production of LPS-induced proinflammatory cytokines, such as TNF-α and IL-1β [30], and increased LPS-induced expression of the anti-inflammatory cytokine IL-10 [31]. Several possible mechanisms have been postulated to explain the anti-inflammatory action of CO. CO modulated mitogen-activated protein kinase (MAPK) pathways, including p38 MAPK and c-jun *N*-terminal kinase pathways [10, 11]. CO causes a general down-regulation of proinflammatory cytokine production through p38 MAPK-dependent pathways and nuclear factor-κB inactivation [30].

3.3. HO-1 against ER Stress. Molecules involved in ER stress response have two opposing functions; adaptive or proapoptotic. ER stress-responsive molecules have an adaptive function in cells that are exposed to mild and transient stresses, whereas these molecules have a proapoptotic function in cells exposed to severe and chronic stress. A study has shown that HO-1 expression was induced in response to ER stress-inducing chemicals, such as thapsigargin, homocysteine and tunicamycin, in smooth muscle cells (SMCs) [32]. Interestingly, exogenous application of the HO-1 byproduct CO inhibited apoptosis induced by ER stress-inducing agents in SMCs, which was associated with the downregulated expression of the proapoptotic proteins. In human endothelial cells, HO-1/CO system also inhibited ER stress-induced apoptosis *via* p38 MAPK-dependent inhibition of the proapoptotic CHOP expression [26]. These studies suggest that HO-1/CO can confer cytoprotection against apoptotic signals originating from ER stress-responsive molecules.

4. HO-1 Inducers and Therapeutic Potentials

Many phytochemicals, which have reported antioxidant and anti-inflammatory properties, could be explored for their potential to reverse oxidative stress, inflammatory stress, and ER stress, which may be finally useful for management of diabetes. HO-1 has been shown to protect against cellular stress-associated physiological disorders on the basis of its rapid up-regulation under various stress conditions and potent physiological regulating properties. Therefore, HO-1 expression has been suggested to have a general adaptive response and enhanced resistance to various stresses [10, 11]. In this regard, pharmacological expression of HO-1 may be a novel therapeutic intervention for diabetes.

4.1. HO-1 Expression. Targeted modulation of HO-1 expression for potential therapeutic interventions requires detailed knowledge of the mechanisms that regulate HO-1 gene expression. The nuclear factor-erythroid 2-related factor 2 (Nrf2) is recognized as a major contributor to the up-regulation of multiple antioxidant defense system in response to various phytochemicals. Nrf2 binds to the antioxidant-responsive element (ARE) or the electrophile-responsive element [10, 11]. ARE has been detected in the promoter or upstream promoter regions of the genes encoding phase II antioxidant enzymes including glutathione *S*-transferase subunits, glutamate-cysteine ligase catalytic and glutamate-cysteine ligase modifier subunits, the thioredoxin and peroxiredoxin families, and NAD(P)H:quinone oxidoreductase [10, 11]. HO-1 is upregulated *via* activation of the Nrf2-ARE pathway. Nrf2 activation is mainly controlled by the cytosolic inhibitor Kelch-like enoyl-CoA-hydratase-associated protein1 (Keap1) [10, 11]. Under normal conditions, Nrf2 is anchored in the cytoplasm through binding to Keap1, which, in turn, facilitates the ubiquitination and subsequent proteolysis of Nrf2. Such sequestration and degradation of Nrf2 in the cytoplasm are mechanisms for the repressive effects of Keap1 on Nrf2. Disruption of the Nrf2-Keap1 complex can result from modification of critical cysteines of Keap1. Numerous stimuli cause disruption of the Nrf2-Keap1 complex *via* modulation of these critical cysteines, which permits subsequent nuclear translocation of free Nrf2 [10, 11].

4.2. Cur Analogues as HO-1 Inducers. Curcuminoids are the active components responsible for the majority of the medicinal properties of turmeric, and there are 3 naturally occurring curcuminoids: Cur, demethoxycurcumin (DMC), and *bis*-demethoxycurcumin (BDMC). Tetrahydrocurcumin (THC) is one of the major metabolites of Cur, and dimethoxycurcumin (DiMC) is one of synthesized Cur derivatives. The chemical structures of Cur analogues are shown in Figure 2. While Cur contains two methoxyl groups at its *ortho*-position, DMC contains only one and BDMC contains none. In comparison with Cur, DiMC contains additional two methoxyl groups instead of two hydroxyl groups, and THC, like Cur, contains two methoxyl groups and two hydroxyl groups but lacks conjugated double bonds in the central seven-carbon chain. Cur has been first reported to induce *in vitro* HO-1 expression through Keap1-Nrf2/ARE pathway in renal epithelial cells [33], which was further confirmed in rat vascular SMCs [34]. It has been shown that the α,β-unsaturated carbonyl group may be an important structure of curcuminoids, because THC, lacking this functional group, was virtually inactive in inducing HO-1 expression [34]. In fact, compounds carrying this reactive group have been reported to induce HO-1 expression through activation of Nrf2 nuclear translocation [35]. It has been noted that three naturally occurring curcuminoids vary in their ability to induce HO-1 expression in human endothelial cells [36]. The levels of HO-1 expression were found to be highest with Cur, followed by DMC and BDMC. Considering that the main difference among the three curcuminoids is the number of methoxyl groups (none for BDMC, one for DMC,

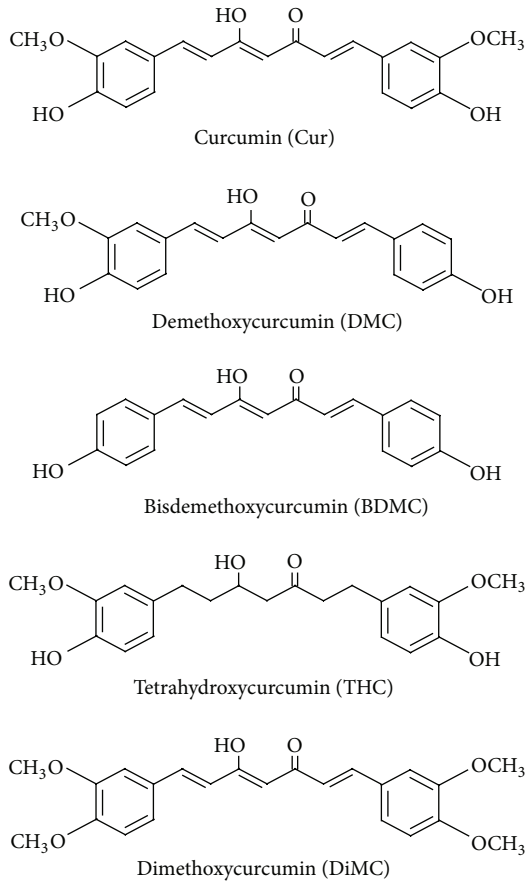

Figure 2: Chemical structures of Cur analogues.

and two for curcumin), the presence of methoxyl groups in the *ortho*-position on the aromatic ring has been suggested to be essential to enhance HO-1 expression [36], and this finding may be useful in designing more efficacious HO-1 inducers. Cur is rapidly metabolized *in vivo* into THC and other reduced forms [37]. Moreover, HO-1-inducing property of Cur is lost when it is reduced to THC [34, 35]. Thus, there is a need to develop Cur analogues with higher metabolic stability than the original Cur. DiMC, one of several synthetic Cur analogues, was reported to have increased metabolic stability in comparison with Cur [38], and, similar to Cur, induced HO-1 expression *via* Nrf2 activation in RAW264.7 macrophages [35]. Recently, a novel water soluble Cur derivative (NCD) has been developed to overcome low *in vivo* bioavailability of Cur and to evaluate its therapeutic effects in rats with diabetes mellitus [39]. Administration of oral NCD or pure Cur to diabetic rats significantly decreased blood glucose levels and increased the plasma insulin, as compared with the diabetic group, and NCD was more effective in such effects than Cur. Oral NCD did not change the plasma glucose levels in the control group, while it significantly increased the plasma insulin in the control group. Interestingly, treatment of diabetic rats receiving oral NCD with the HO-1 inhibitor zinc protoporphyrin resulted in a significant increase in the plasma glucose level and a significant decrease in insulin levels, when compared with the diabetic group receiving oral NCD only. Administration of oral NCD or pure Cur significantly increased the HO-1 expression level in the pancreatic tissues of the diabetic group, as compared with controls. Thus, it was suggested that the hypoglycemic action of Cur might be mediated through HO-1 expression.

5. Conclusions

There may be the integration of oxidative stress, inflammatory stress, and ER stress in the pathogenesis of diabetes. Depending on the cell type and physiological process, either oxidative stress, inflammatory stress, or ER stress may be more prominent or upstream of the others. However, these signaling pathways may interact and be ultimately integrated in the pathogenesis of diabetes. Given the integration of oxidative stress, inflammatory stress, and ER stress, targeting only one of them may not be effective in controlling disease pathogenesis. As abovementioned, HO-1 has its potential ability to modulate oxidative stress, inflammatory stress, and ER stress, and this has generated immense interest in HO-1 as a therapeutic target (Figure 1). Metalloporphyrins, such as CoPP and hemin, which are prototypical inducers of HO-1 and are commonly used in experimental cell culture and animal models, do not seem to be applicable for clinical interventions, because they lack cell-specificity and are severely toxic when it is used for long periods of time. Naturally occurring phytochemicals ameliorate the risk factors that lead to the development of diabetes, but the mechanisms of their actions remain to be established. Besides their capacity to directly quench ROS, some of them, such as Cur, reduce the incidence of diabetes *via* HO-1 expression [3–9], which allows them to be considered as HO-1 inducers that may provide an alternative strategy for controlling the initiation and progression of diabetic diseases. However, their introduction into the clinical setting may be hindered largely by their poor solubility, rapid metabolism, or a combination of both, ultimately resulting in low therapeutic concentrations at the target site. To overcome the bioavailability, advanced drug delivery systems, designed to provide localized or targeted delivery of these agents, may provide a more viable therapeutic option in the treatment of diabetes.

Conflict of Interests

The authors confirm that there is no known conflict of interests associated with this publication and that there has been no significant financial supports for this work that could have influenced its outcome. They also confirm that they do not have a direct financial relation with any commercial identity mentioned in their paper that might lead to a conflict of interests for any of the authors.

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Research Article

Protective Effect of Spin-Labeled 1-Ethyl-1-nitrosourea against Oxidative Stress in Liver Induced by Antitumor Drugs and Radiation

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This study was carried out to investigate possible protection effect of 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU), synthesized in our laboratory, against oxidative liver injuries induced in mice treated by antitumor drugs: doxorubicin (DOX), bleomycin (BLM), or gamma irradiation (R). Specifically, alterations in some biomarkers of oxidative stress, such as lipid peroxidation products measured as malondialdehyde (MDA) levels and activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were studied in liver homogenates isolated from tumor bearing C57 black mice after i.p. treatment with solutions of DOX (60 mg/kg), BLM (60 mg/kg), or after total body gamma-irradiation with a single dose of 5 Gy. The same biomarkers were also measured after i.p. pretreatment of mice with SLENU (100 mg/kg). Statistical significant increased MDA levels and SOD and CAT enzymes activities were found in the liver homogenates of tumor bearing mice after alone treatment with DOX or gamma-irradiation compared to the control mice, while these parameters were insignificantly increased after BLM administration compared to the same controls.

1. Introduction

Modern chemotherapy, along with surgery and radiation therapy, is still the most efficient method of cancer treatment. The final common pathway in the mechanisms of action of ionizing radiation and many chemotherapeutic agents include alterations of DNA and the production of reactive oxygen species (ROS) [1, 2]. In particular, double-strand breaks have a major impact on cell killing after irradiation. The increased production of ROS, however, could be a reason for many dangerous side effects that sometimes hamper the therapy and may lead to serious or even fatal organ dysfunctions.

Among the anticancer drugs, doxorubicin and bleomycin have been used for the treatment of many malignant tumors. Although these drugs belong to different classes, doxorubicin is an anthracycline glycoside antibiotic, whereas bleomycin is a glycosylated peptide antibiotic, they share some properties. Thus, ROS were shown to be involved in the toxicity of both doxorubicin and bleomycin [3, 4]. Also, chronic organ toxicity frequently develops upon administration of cumulative doses of both drugs. Finally, interactions of both drugs with iron are considered to be of importance in exerting their deleterious effects on healthy tissues as well as in their antineoplastic activity [5, 6]. Bleomycin has been used for the treatment of germ cell tumors, lymphomas, Kaposi's sarcomas, and so forth. Bleomycin is considered radiomimetic and oxidative DNA-cleaving reagent [7]. The clinical usefulness of BLM is restricted, since it has several acute and chronic side effects. The most serious complications of BLM are pulmonary fibrosis and impaired lung function. Minor important adverse effects are myelosuppression, nauseas, vomiting, allergic reactions, mucositis, alopecia, erythema, hyperkeratosis, hypopigmentation, skin

ulceration, and acute arthritis [8]. Hepatotoxicity is also minor and reversible [4, 9]. Doxorubicin possesses a potent and broad-spectrum antitumor activity against a variety of human solid tumors and hematological malignancies. However, its use in chemotherapy has been limited largely due to its diverse toxicities. Reactive oxygen species, generated by the interaction of doxorubicin with iron, can damage cellular systems, with the most serious adverse effect being life-threatening heart damage. Other tissues, like the kidneys, brain, liver, and the skeletal muscles, are also affected by DOX [10, 11]. Chemotherapy with DOX can cause liver abnormalities such as ascites, hyperbilirubinemia, reactivation of hepatitis B, and thrombocytopenia leading to fatalities [12–14].

At least 50 percent of all cancer patients receive radiotherapy at some stage during the course of their illness. Radiotherapy is currently used to treat localized solid tumors, such as cancers of the skin, brain, breast, or cervix, and can also be used to treat leukemia and lymphoma [15, 16]. However, a number of patients undergoing radiation therapy experience a range of side effects, which may lead to an interruption of treatment or limiting the dose of radiation. A growing body of evidence appears to support the hypothesis that oxidative stress might serve to drive the progression of radiation-induced toxic side effects [17–19]. Free radicals are considered to be the common mediator of DNA damage after ionizing radiation. Radiation's effects on normal tissues occur predominantly in slowly growing tissues such as the lungs, liver, kidneys, heart, and central nervous system [15].

Strategies to attenuate drugs and radiation toxicity include dosage optimization, synthesis, and the use of analogues having lower toxicity or a combined therapy with antioxidants. Clinical and experimental trials have been directed toward employing various antioxidant agents to ameliorate drug- and radiation-induced liver damage. The most promising results come from the combination of the drug delivery together with an antioxidant in order to reduce oxidative stress. Although a number of studies have examined the protective effects of antioxidants such as vitamins C and E, carotenoids, and selenium, these studies have not provided consistent evidence in favor of hepatotoxic effects of the anthracyclines and radiation [20]. Other compounds such as erdosteine, cystathionine, and catechin might also prevent oxidative liver injury induced by these antitumor drugs and radiation [21, 22].

Stable nitroxyl radicals such as 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) have been shown to function as superoxide dismutase (SOD) mimics and to protect mammalian cells against superoxide and hydrogen peroxide-mediated oxidative stress and radiation-induced cytotoxicity [23]. Reduced toxicity and increased antineoplastic properties were achieved when nitroxyl (aminoxyl) groups were introduced in chemical structure of certain antitumor drugs [24, 25]. This finding encourages us to synthesize a number of spin-labeled analogues of the anticancer drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). Some of these compounds showed advantages over CCNU, having lower toxicity and higher anticancer activity against some experimental tumor models [26, 27]. By EPR method, we have shown that spin-labeled nitrosoureas and their precursor 4-amino TMPO can scavenge $^{\bullet}O_2^-$ and so exhibit high superoxide-scavenging activity (SSA) [28]. Moreover, by our studies, we have demonstrated beneficial effects of SLENU, recently synthesized in our laboratory, analogue of the antitumor drug CCNU, and vitamin E as positive control on CCNU-free radical-induced oxidative injuries in rat blood and in liver of mice [29, 30].

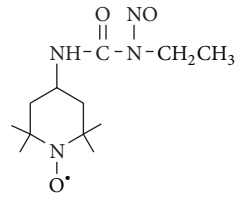

Figure 1: Chemical structure of 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU).

Therefore, the aim of the present study was to investigate whether, pretreatment with spin-labeled nitrosourea SLENU (Figure 1) possessing high SSA would decrease liver oxidative stress injuries in mice induced by application of antitumor drugs or gamma irradiation. To achieve the ultimate goal of this research, we investigated the levels of lipid peroxidation and activities of antioxidant defense enzymes superoxide dismutase (SOD) and catalase (CAT) in liver homogenates of tumor bearing mice treated by the antitumor drugs doxorubicin, bleomycin, or after total body irradiation alone and compared to the levels of the same parameters measured after pretreatment with SLENU.

2. Materials and Methods

2.1. Drugs and Chemicals. Bleomycin and Farmorubicin were obtained from Bristol Myers Squibb, Wallingford, CT, USA. Buttermilk xanthine oxidase, trolox, SULF (sulfanilamide), NEDD (N-(1-naphthyl) ethylenediamine dihydrochloride), and VCl_3 were obtained from Fluka (Germany). TMPO was purchased from Aldrich (Milwaukee, USA). SLENU was synthesized according to Gadjeva and Koldamova [31]. The test compounds were dissolved ex tempore: first step in Tween and second step in saline.

2.2. Experimental Animals. All procedures performed on animals were done in accordance with guidelines of the Bulgarian government regulations and were approved by the authorities of Trakia University. The animals were housed in plastic cages, fed a normal laboratory diet and water ad libitum.

The study was carried out on 142 C57 black mice (bred in the Laboratory of Oncopharmacology, National Cancer Institute, Sofia), with average weight of 18–22 g, divided into groups of 6 animals per group (equal number of the two sexes).

2.3. Experimental Design. The blood for the analysis was taken by a heart puncture after opening the thoracic region. The venous blood samples were divided into portions. The serums were used for an analysis of enzymatic activities and the level of NO. Mice were sacrificed by cervical decapitation at 1 hour after administration of the drugs or gamma irradiation. Livers were removed and kept on ice until homogenization on the same day. The samples were first washed with deionized water to separate blood and then homogenized. The tissue homogenates were centrifuged at 15 000 rpm for 10 minutes, 4°C and the final supernatants were obtained. They were used for determination of lipid peroxidation and the activities of superoxide dismutase and catalase.

2.3.1. Drug Treatment. On day 0, mice were inoculated i.p. with 10^5 tumor cell suspension from lymphoid leukemia L1210 in saline in volume of 0.5 mL. On day 3, Bleomycin (60 mg/kg), Farmorubicin (60 mg/kg), in accordance with LD50 of the drugs, spin-labeled nitrosourea SLENU (100 mg/kg), and combinations of them were administrated i.p. in a single injection in volume 0.01 mL per body weight, as 10% Tween solutions in accordance with the routine methods described in the literature [32].

2.3.2. Irradiation. Total-body irradiation of mice was performed with an orthovoltage Philips RT-250 irradiator, 225 kVp X-ray source, operating at 15 mA and filtered with 0.2 cm copper. Mice were exposed to 5 Gy total-body γ-irradiation at a dose rate of 2,52 cGy/s in the absence or presence of injected SLENU (100 mg/kg), 10 min after administration. All animals were weighed prior to irradiation. After irradiation animals were returned to the animal facility.

2.4. Investigation of Oxidative Stress Parameters

2.4.1. Analysis of Lipid Peroxidation in Liver. Basal levels of lipid peroxidation as indicated by thiobarbituric acid-reactive substances (TBARS) were determined using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products [33]. In the TBARS assay, 1 mL of the supernatant, 1 mL of normal saline, and 1 mL of 25% trichloroacetic acid (TCA) were mixed and centrifuged at 2000 rpm for 20 minutes. One mL of protein-free supernatant was taken, mixed with 0.25 mL of 1% TBA and boiled 1 h at 95°C. After cooling, the absorbance of the pink color of the obtained fraction product was read at 532 nm.

2.4.2. Measurement of Antioxidant Enzymes Activities in Liver. Total SOD activity was determined by the xanthine/xanthine-oxidase/nitroblue tetrazolium (NBT) method according to Sun et al. [34], with minor modification. Superoxide anion radical (O_2^-) produced by xanthine/xanthine-oxidase system reduces NBT to formazan, which can be assessed spectrophotometrically at 560 nm. SOD competes with NBT for the dismutation of $^{\bullet}O_2^-$ and inhibits its reduction. The level of this reduction is used as a measure of SOD activity. The total SOD activity is expressed in units/mg of protein, where one unit was equal to SOD activity that causes 50% inhibition of the reaction rate without SOD.

The assay of CAT activity was according to Beers Jr. and Sizer [35]. Briefly, hydrogen peroxide (30 mM) was used as a substrate and the decrease in H_2O_2 concentration at 22°C in a phosphate buffer (50 mM, pH 7.0) was followed spectroscopically at 240 nm for 1 min. The activity of the enzyme was expressed in units per mg of protein, and 1 unit was equal to the amount of an enzyme that degrades 1 μM H_2O_2 per minute.

2.4.3. Measurement of $NO^{\bullet}$ in Serum. Serum nitric oxide was measured in terms of its products, nitrite and nitrate, by the method of Griess, modified by Miranda et al. [36]. This method is based on a two-step process. The first step is the conversion of nitrate to nitrite using vanadium (III) and the second is the addition of sulphanilamide and N (-naphthyl) ethylenediamine (Griess reagent). This converts nitrite into a deep-purple azo compound, which was measured colorimetrically at 540 nm. Nitric oxide products were expressed as μM.

2.5. Estimation of Serum Transaminases sGPT and sGOT. The liver function was evaluated with serum levels of glutamate oxaloacetate transaminase sGOT and glutamate pyruvate transaminase sGPT. The determination of sGOT and sGPT was based on the fact that phenylhydrazone, which produced after incubation the substrate with the enzyme, was measured spectrophotometrically [37]. The amount of phenyl hydrazone formed was directly proportional to the enzyme quantity.

2.6. Statistical Analysis. The data are expressed as a mean ± SE. Student's t-test was used to determine the statistical differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of SLENU on sGPT and sGOT Levels. In the present study, the liver function was evaluated with serum levels of glutamate oxaloacetate transaminase sGOT and glutamate pyruvate transaminase sGPT, which were measured in the serum as markers of cellular injury. There were not significant changes of the levels of sGPT and sGOT between healthy and tumor bearing control mice. The levels of sGPT and sGOT were increased but not significantly ($P > 0.05$) after i.p. administration of a single dose of BLM (60 mg/kg) in tumor bearing mice. However, there was dramatic increase in the enzymes levels of sGPT and sGOT after i.p. administration of DOX in dose (60 mg/kg) and gamma irradiation with a single dose of 5 Gy in tumor bearing mice, compared to the untreated cancer control groups ($P < 0.00001$). When mice were pretreated with SLENU i.p. dose (100 mg/kg), 30 min. prior to DOX and gamma-irradiation, a statistical significant reduction was found in the levels of sGPT and sGOT compared to the groups treated with DOX or gamma irradiation alone ($P < 0.0001$) (Table 1).

Table 1: The influence of i.p. administration of SLENU on sGPT and sGOT liver transaminases in BLM, DOX, and R-treated mice.

Compound	sGOT (U/L)	sGPT (U/L)
Controls (health)	60.20 ± 2.60	31.00 ± 0.44
Controls (tumor)	63.27 ± 1.43	36.15 ± 1.74
BLM (60 mg/kg)	78.90 ± 4.10	44.23 ± 0.81
DOX (60 mg/kg)	177.87 ± 2.30*	142.33 ± 1.21*
R	181.80 ± 3.12*	134.56 ± 0.90*
SLENU (100 mg/kg)	62.88 ± 2.04	39.27 ± 1.98
BLM (60 mg/kg) + SLENU (100 mg/kg)	66.50 ± 1.40	41.20 ± 0.62
DOX (60 mg/kg) + SLENU (100 mg/kg)	80.30 ± 1.27#	49.89 ± 1.02#
R (60 mg/kg) + SLENU (100 mg/kg)	77.45 ± 1.13#	37.10 ± 0.77#

Data are expressed as mean ± SE; * $P < 0.00001$ versus tumor controls; # $P < 0.0001$ versus corresponding DOX or R-treated mice.

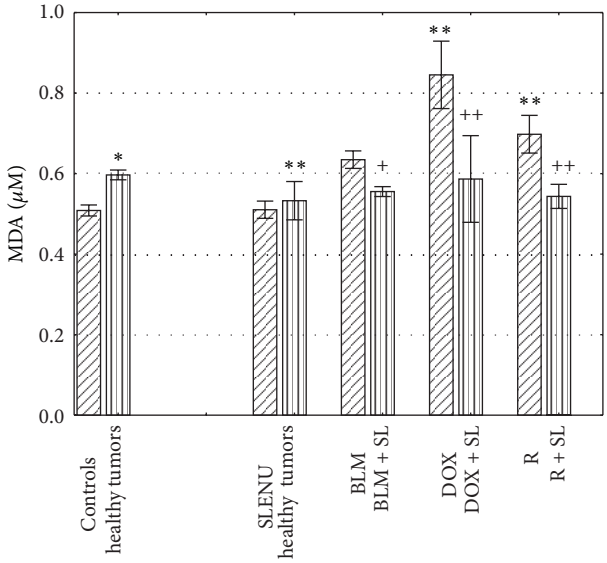

Figure 2: Lipid peroxidation in liver homogenates isolated from mice 1 hour after i.p. administration of BLM, DOX, and R alone and in combination with SLENU. Values are expressed as mean ± SE. * $P < 0.001$ versus health controls; ** $P < 0.0001$ versus tumor controls + $P < 0.01$ versus group with BLM administrated alone; ++ $P < 0.0001$ versus group with DOX and R administrated alone.

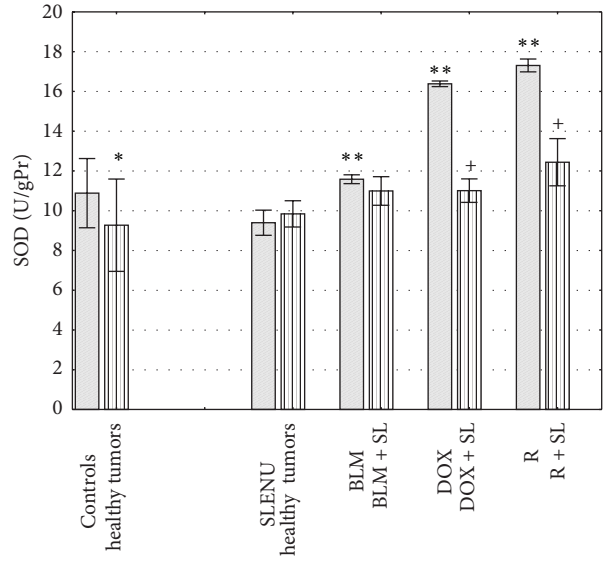

Figure 3: SOD activity of liver homogenates isolated from mice at 1 hour after administration of BLM, DOX, and R alone or in combination with SLENU. Values are expressed as mean ± SE. * $P < 0.001$ versus health controls; ** $P < 0.0001$ versus tumor controls; + $P < 0.05$ versus group treated with DOX alone; ++ $P < 0.0001$ versus group with DOX and R administrated alone.

3.2. Effect of SLENU on MDA Level and Antioxidant Enzymes SOD and CAT in Liver. The levels of lipid peroxidation in liver homogenates isolated from mice treated with BLM, DOX, and R alone and in combination with SLENU are shown in Figure 2. It was found that the levels of MDA were significantly increased in tumor bearing mice compared to the healthy controls (0.606 μM versus 0.508 μM, $P < 0.001$). No significant difference, compared to the healthy controls, was observed in the groups of either tumor bearing or healthy mice treated with SLENU (mean 0.488 U/gPr and 0.426 U/gPr, $P > 0.05$). One hour after administration of BLM, DOX and R, the levels of MDA were significantly increased in liver homogenates isolated from tumor bearing mice treated with DOX, and R, compared to the group of tumor controls (mean 0.893 μM and 0.698 μM, $P < 0.0001$) and not significantly increased in liver homogenates isolated from tumor bearing mice treated with BLM (mean 0.625 μM, $P > 0.05$). Combined application of BLM and SLENU led to a decrease in the level of MDA compared to the level when BLM was administrated alone (mean 0.561 μM, $P < 0.01$). However, combinations of either DOX or R with SLENU led to a strong decrease in the levels of MDA, compared to the levels when DOX and R were administrated alone (mean 0.569 μM and 0.543 μM, $P < 0.0001$); the levels of the former were close to those obtained from SLENU when administered alone.

As can be seen from the data represented in Figure 3, the activities of SOD in liver homogenates isolated from tumor bearing control mice at 1 h were significantly decreased compared to SOD activities of liver homogenates, isolated from healthy controls (mean 8.472 U/gPr versus 10.882 U/gPr, $P < 0.001$). No significant difference, compared to the healthy

controls, was observed in the groups of either tumor bearing or healthy mice treated with SLENU (mean 9.826 U/gPr and 8.688 U/gPr, $P > 0.05$). After treatment with BLM, DOX, and R alone, SOD activities of liver homogenates from tumor bearing were found to be significantly higher than those of the tumor bearing controls (mean 11.583 U/gPr, 16.213 U/gPr, and 17.306 U/gPr, $P < 0.0001$, resp.). However, a combined application of BLM, DOX, and R with SLENU had lower SOD activities for tumor bearing mice compared to the groups of tumor bearing mice treated with BLM, DOX, and R alone and was close to the healthy controls. A combined application of BLM and SLENU led to a decrease but not significant in the level of SOD compared to that of BLM administrated alone in tumor bearing mice (mean 10.924 U/gPr, $P > 0.05$). Moreover, in tumor bearing mice after administration of the combination of either DOX or R with SLENU, SOD activities were significantly decreased compared to those of DOX and R administrated alone (mean 10.698 U/gPr and 12.251 U/gPr, $P < 0.0001$) and were close to those of the healthy controls.

Figure 4 represents the activity of the antioxidant enzyme CAT in liver homogenates isolated from healthy and tumor bearing mice. The activity of CAT in tumor bearing control mice was significantly increased compared to the healthy controls (mean 37.428 U/gPr versus 28.059 U/gPr, $P < 0.0001$). The activity of CAT in the liver homogenates after treatment of either healthy or tumor bearing mice with SLENU was not significantly higher compared to the healthy controls (mean 32.402 U/gPr and 31.218 U/gPr, $P > 0.05$). One hour after application of BLM, DOX or R the activities of CAT in tumor bearing mice were increased compared to the tumor bearing controls (mean 39.380 U/gPr, $P > 0.05$; 63.667 U/gPr and 55.590, $P < 0.0001$). However, pretreatment with SLENU and following application of BLM, DOX, or R, led to significantly decreased levels of the antioxidant enzyme CAT compared to the groups of tumor bearing mice with BLM, DOX, or R administrated alone (mean 37.199 U/gPr, $P < 0.001$; 30.409 U/gPr and 33.255 U/gPr, $P < 0.00001$). Moreover, CAT activities in all combinations were found to be close to those of the controls.

3.3. Effect of SLENU on Total End Products of NO_2^- and NO_3^- in the Serum. Figure 5 shows the levels of $NO^\bullet$ expressed as total end products of NO_2^- and NO_3^-. The levels of $NO^\bullet$ were found to be increased but not significantly in tumor bearing mice compared to healthy controls (mean 5.781 μM versus 1.373 μM, $P > 0.05$). Tumor bearing mice treated with BLM, DOX or exposed to gamma irradiation had remarkably increased levels of $NO^\bullet$ compared to the tumor controls (mean 35.252 μM, 33.915 μM, and 30.153 μM, $P < 0.00001$, resp.). It is interesting that mice treated with SLENU had also significantly higher level of $NO^\bullet$ than that of tumor controls (mean 44.088 μM, $P < 0.00001$) and also than mice treated with BLM, DOX or exposed to gamma radiation alone, ($P < 0.0001$). Moreover, the levels of $NO^\bullet$ for the combinations of BLM, DOX, or gamma irradiation with SLENU were not significantly different from those in mice treated with SLENU alone (mean 40.088 μM, 40.187 μM, and 39.081 μM, $P > 0.05$, resp.).

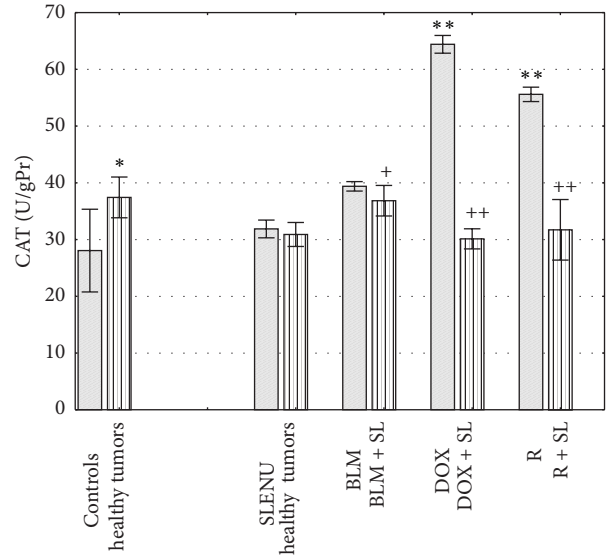

Figure 4: CAT activity of liver homogenates isolated from mice 1 hour after administration of BLM, DOX, or R alone or in combination with SLENU. Values are expressed as mean ± SE. $^*P < 0.0001$ versus health controls; $^{**}P < 0.0001$ versus tumor controls; $^+P < 0.001$ versus group treated with BLM alone; $^{++}P < 0.00001$ versus groups treated with DOX and R alone.

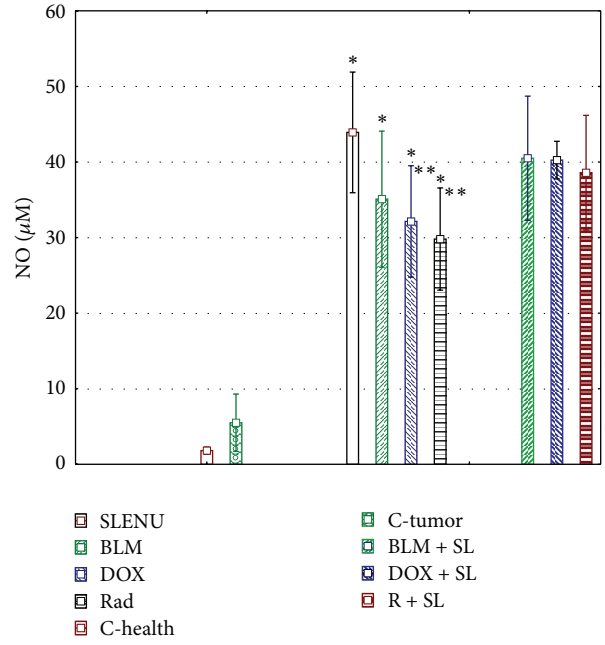

Figure 5: $NO^\bullet$ expressed as total end products of NO_2^- and NO_3^-. $^*P < 0.00001$ versus controls; $^{**}P < 0.0001$ versus controls.

4. Discussion

The current study was undertaken to evaluate the protective effect of the spin-labeled nitrosourea SLENU against oxidative stress induced in liver of mice treated by antitumor drugs DOX, BLM, or gamma irradiation.

It has already been reported that the stable nitroxyl radical Tempol and other analogues represent a new class of non-thiol-containing radiation protectors that may be useful in elucidating the mechanisms of radiation-induced cellular damage and may have broad applications in protecting against oxidative stress. Further, bearing in mind formerly reported facts by us: (1) an excellent expressed superoxide anion scavenging activity (SSA) of the spin-labeled nitrosourea SLENU and (2) beneficial effects of SLENU on CCNU-induced oxidative stress, we have tried to explain the protective effect of the spin-labeled nitrosourea SLENU on oxidative stress induced by DOX, BLM, and gamma irradiation with possible involvement of free radical mechanisms.

Serum enzyme levels of sGOT and sGPT were measured as primary and specific markers of liver injury. Our results showed increase from three to four times in the levels of sGOT and sGPT for mice treated with either DOX or R alone, compared to the untreated control group, and a slight increase for BLM treated group, compared to the same untreated control group. These findings were in accordance with other authors [9, 10, 21]. After pretreatment with SLENU, the activities of these enzymes were decreased to values similar to those of the controls.

The levels of lipid peroxidation products (MDA) and the activities of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), in liver homogenates were used as indicatives of oxidative liver injury. To evaluate the oxidative status in liver of treated mice, all measurements were carried out at the 1st hour after the treatment. This time was chosen taking into account our last electron spin resonance (ESR) study by which it was demonstrated that after SLENU i.p. administration, its maximal concentration in lungs, brain, liver, and spleen was reached at 30 min and completely was absent within 90 min in all tissues studied [31].

The results of the present study showed that the levels of MDA and the activities of the enzymes SOD and CAT were found to be significantly changed in tumor bearing mice compared to the healthy controls. This suggests an increased oxidative stress and imbalance in the antioxidant defense in non-treated tumor bearing mice as a consequence of abnormality in antioxidative metabolism due to the cancer process.

Administration of antineoplastic agents during cancer chemotherapy results in a much greater degree of oxidative stress than that induced by cancer itself. The high level of oxidative stress during chemotherapy may overcome the antioxidant defenses of cancer cells, resulting in lipid peroxides production and interfering with antineoplastic activity [20, 38]. Our results demonstrated that administration of the antitumor drug DOX by i.p. route or total body gamma irradiation caused a much greater degree of oxidative stress than that induced by cancer itself. Immediately 1 hour after treatment with DOX or after total body irradiation, liver homogenates of tumor bearing mice had higher levels of lipid peroxidation products compared to the tumor bearing controls. It was accompanied by disturbance in the antioxidant enzyme defense-increased SOD and CAT activities. After treatment with DOX or after total body irradiation, the oxidative stress and the imbalance of antioxidant enzyme system significantly progress. This disturbance might be due to the augmented generation of toxic reactive oxygen species (ROS) in the liver induced by DOX and irradiation. In addition, these free radicals also mediate oxidation of other cellular molecules and have an important role in the pathogenesis of drug and radiation-induced liver abnormalities. Increased levels of oxidative stress enzymes (SOD, GSH-Px, GR, and CAT) were observed and confirmed in DOX-induced rats [39]. The disturbance in oxidant-antioxidant systems results in tissue injury, which is demonstrated with lipid peroxidation and protein oxidation in the tissue. Several studies have shown that the combination of the inflammatory process, free radical oxidative stress, and lipid peroxidation is frequently associated with liver damage, induced by toxic agents such as DOX [21, 22]. Increased MDA levels and SOD and CAT enzymes activities were found in the liver homogenates of tumor bearing mice after alone treatment with BLM compared to the control mice. Although those increases were not statistically significant, they positively affected by pretreatment of SLENU. This finding additionally confirmed that, at our experimental conditions, BLM acts as a reactive oxygen species- (ROS-) generating drug in liver tissues of mice. Even though the liver is not susceptible to BLM toxicity, apparently some markers of oxidative stress are highly sensitive to this drug. Similar results were previously obtained by other authors, who reported that the hepatic microsomal mixed-function oxidase system is highly sensitive to BLM [4]. The effect of BLM as a ROS-generating antitumor drug was evaluated on antioxidant enzymes and the electron transport system in different cellular fractions of liver in rats [40]. The authors reported that the induced antioxidant enzyme activities in BLM-treated rats may be a response to excessive free radical generation due to BLM metabolism in the animals.

Another indirect proof for involvement of ROS in drug- and radiation-induced toxicity is the overcome of the oxidative stress by adding typical antioxidants. For example, vitamin E, via its robust free radical scavenging effect, prevents lipid peroxidation and therefore inhibits the hepatotoxic effects of doxorubicin [13]. In order to evaluate the effect of SLENU on BLM, DOX, and R-induced oxidative stress, the tissue levels of MDA and the activities of antioxidant enzymes SOD and CAT were measured after treatment with the combinations of BLM, DOX, or R with SLENU. MDA levels were decreased and antioxidant enzymes SOD and CAT activities were normalized to levels close to the controls. Therefore, with the present study, it was prove a complete overcome of the oxidative stress induced by BLM, DOX, and R when the typical antioxidant SLENU possessing high SSA was added. These results propose that SLENU might be a potential hepatoprotector in doxorubicin, bleomycin, and radiation-induced hepatotoxicity.

Based on this finding, we have hypothesized that if BLM, DOX, and R could generate $^{\bullet}O_2^-$ and $^{\bullet}NO$ *in vivo*, it might contribute to tissue $ONOO^-$ and $^{\bullet}OH$ production, and these could be a reason for the oxidative liver injury (increase in

MDA level and alteration in SOD and CAT activities) by the following reactions:

$$\begin{aligned} {}^{\bullet}NO + O_2^{-} &\longrightarrow ONOO^{-} \\ ONOO^{-} + H^{+} &\longrightarrow ONOOH \\ ONOOH &\longrightarrow {}^{\bullet}OH + {}^{\bullet}NO_2 \\ ONOOH &\longrightarrow NO_2^{-} + H^{+} \end{aligned} \quad (1)$$

When mice were pretreated with SLENU, a complete overcome of the oxidative stress in liver homogenates, which is due to DOX, BLM, or R, was observed. A chemopreventive effect of nitroxides such as Tempol was reported by several authors [2, 10, 23]. Authors demonstrated that nitroxides at nontoxic concentrations are effective as *in vitro* and *in vivo* antioxidants, when oxidation is induced by superoxide, hydrogen peroxide, organic hydroperoxides, ionizing radiation, or specific DNA-damaging anticancer agents. The authors explained the protection of oxidative damage by nitroxides through several possible chemical explanations: (1) SOD-mimicking action; (2) oxidation of reduced metals that have potential to generate site-specific ${}^{\bullet}OH$ radicals; (3) termination of free radical chain reactions induced by alkyl, alkoxyl, alkylperoxyl radical species, and detoxifying drug-derived radicals; and (4) detoxification of hypervalent toxic metal species such as ferryl and cupryl ions.

By EPR studies, we have established that the spin-labeled nitrosourea derivatives, such as SLENU, could successfully scavenge ${}^{\bullet}O_2^{-}$ by exhibiting high SSA [28]. We also showed that the mechanism of SSA activity was through redox cycling between nitroxide and its corresponding hydroxylamine moiety, according to the following proposed equations:

$$\begin{aligned} N\text{–}O^{\bullet} + {}^{\bullet}O_2^{-} + H^{+} &\xrightarrow{k_r} N\text{–}OH \\ N\text{–}OH + {}^{\bullet}O_2^{-} + H^{+} &\xrightarrow{k_o} N\text{–}O^{\bullet} + H_2O_2 \end{aligned} \quad (2)$$

where k_r and k_o are second-order rate constants for the reduction of nitroxide and oxidation of hydroxylamine by superoxide, respectively.

The nontoxic effect of the spin-labeled nitrosourea SLENU and its ability to reverse the BLM, DOX, and R-induced oxidative stress in our study have led us to propose the following hypothesis. The nitroso group in the spin-labeled nitrosourea SLENU may lead to the generation of ${}^{\bullet}NO$, when SLENU is used alone or jointly with BLM, DOX, and R. However, the nitroxyl-free radical moiety incorporated only in the spin-labeled compounds might successfully compete with the self-generated ${}^{\bullet}NO$ and that produced by BLM, DOX, and R in the scavenging of ${}^{\bullet}O_2^{-}$. This effect could prevent the formation of highly toxic species such as $ONOO^{-}$ and ${}^{\bullet}OH$ and at the same time could increase the level of ${}^{\bullet}NO$. In this regard, our present results are consistent with the notion that the protective effects of SLENU are due to both SSA and its increased release of ${}^{\bullet}NO$.

In our study, serum levels of nitrite (NO_2^{-}) and nitrate (NO_3^{-}) were used to estimate the level of ${}^{\bullet}NO$ formation, since ${}^{\bullet}NO$ is highly unstable and has a very short half-life. We observed significantly higher ${}^{\bullet}NO$ end products in the plasma of mice treated with BLM, DOX, R, and SLENU alone and also in mice treated with the combination of either the drugs or gamma irradiation with SLENU. These results were in agreement with the results reported by other authors. Gurujeyalakshmi et al. reported increase in NO levels as a result from BLM-induced increases in iNOS message and iNOS protein [41]. Irradiated cells produce more NO in response to either IFN-gamma or LPS, and the increase is mediated by induction of TNF-alpha [17, 42]. Several *in vitro* studies have demonstrated the protective effect of ${}^{\bullet}NO$ in oxidative injury, both in the generalized case and in hepatocytes. Rubbo et al. [43] suggest that ${}^{\bullet}NO$ may act as a primary antioxidant in biological systems by limiting lipid peroxidative chain propagation. Using a model system, authors demonstrated that ${}^{\bullet}NO$ is a potent terminator of radical chain propagation and that ${}^{\bullet}NO$ inhibits peroxynitrite-dependent lipid peroxidation reactions.

5. Conclusions

In view of these facts, we can conclude that the increase in oxidative stress markers and the concomitant change in antioxidant levels indicate the role of oxidative stress in BLM, DOX, and R-induced oxidative liver injuries. Moreover, pretreatment with SLENU shows a protective impact against BLM, DOX, and R-induced oxidative stress and liver injury by scavenging of ${}^{\bullet}O_2^{-}$ and increased ${}^{\bullet}NO$ release. Further studies are, however, needed to clarify the effect of these combinations in antitumor chemotherapy.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Antioxidant Properties of Brazilian Tropical Fruits by Correlation between Different Assays

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Four different assays (the Folin-Ciocalteu, DPPH, enzymatic method, and inhibitory activity on lipid peroxidation) based on radically different physicochemical principles and normally used to determine the antioxidant activity of food have been confronted and utilized to investigate the antioxidant activity of fruits originated from Brazil, with particular attention to more exotic and less-studied species (jurubeba, *Solanum paniculatum*; pequi, *Caryocar brasiliense*; pitaya, *Hylocereus undatus*; siriguela, *Spondias purpurea*; umbu, *Spondias tuberosa*) in order to (i) verify the correlations between results obtained by the different assays, with the final purpose to obtain more reliable results avoiding possible measuring-method linked mistakes and (ii) individuate the more active fruit species. As expected, the different methods give different responses, depending on the specific assay reaction. Anyhow all results indicate high antioxidant properties for siriguela and jurubeba and poor values for pitaya, umbu, and pequi. Considering that no marked difference of ascorbic acid content has been detected among the different fruits, experimental data suggest that antioxidant activities of the investigated Brazilian fruits are poorly correlated with this molecule, principally depending on their total polyphenolic content.

1. Introduction

It is known that the consumption of fruit and vegetable reduces the incidence of cardiovascular and cerebrovascular diseases, stroke, cancer, and ageing related disorders [1–3]. This effect is attributed to the presence in fruit and vegetables of antioxidants able to preserve the correct balance oxidants/antioxidants, in which upset due to an overproduction of oxygen reactive species (ROS) can lead to the so-called "oxidative stress" [4–6].

Substantial damages have been observed when ROS interact with DNA, membrane lipids, and proteins [7–10]. ROS are involved in the carcinogenic stages of initiation, promotion, and progression [11]; they play an important role in the development of cardiovascular diseases such as ischemic injury, arteriosclerosis, hypertension, cardiomyopathies, congenital heart diseases, and stroke; they may be a causal factor of neurological disorders such as Alzheimer's and Parkinson's diseases [12].

Antioxidant substances represent one of the most important defense mechanisms against free radicals, but the only endogenous antioxidant molecules cannot be effective enough to counteract the injuries caused by ROS, particularly in the current times, where lifestyles based on smoke, drugs, alcohol, unbalanced diet, pollution, incorrect exposure to solar radiation, and so forth can facilitate free radicals formation. For this reason increasing the intake of dietary antioxidant is of great importance to enjoy good health, as evidenced by studies on food characterized by high antioxidants content [13].

Unfortunately, no reliable biomarker of antioxidant activity is available up to now [14, 15] because ROS injuries are mediated by different radical and nonradical species which show different physicochemical characteristics and reaction mechanism affecting reactivity, selectivity, partition in aqueous and lipid phase, and so forth [16]. In literature many experimental methods are reported to determine a generic

antioxidant activity of a compound, but results obtained by different investigations are frequently contradictory [17].

The aim of this work is to compare of the results obtained by four different methods usually employed to measure antioxidant properties, that is, reducing capacity by the Folin-Ciocalteu assay, radical scavenging ability towards 2,2′-diphenyl-1-picrylhydrazyl (DPPH method), inhibitory ability on peroxidation of linoleic acid (LA), and total phenolic determination by the enzymatic method [18] in order to (i) verify possible correlations between the results obtained and (ii) obtain more reliable results avoiding possible measuring-method linked mistakes.

These assays were applied to a series of Brazil fruits, with particular interest in the more exotic and less studied species. In fact, information on the nutritional values of the most exotic species of tropical fruits are limited: some studies [19–22] provide evidence for the high antioxidant capacity and significant amounts of flavonoids and vitamin C for the most common Brazilian fruits as mango [23], starfruit [19], and avocado [24], but no data are reported for more exotic fruits, like pitaya, jurubeba, siriguela, and pequi, some of which native peoples utilize in popular medicine.

The results obtained by these measurements were compared with each other and with those obtained by Italian soft fruits known for their antioxidant activity [25, 26]. Furthermore, to discriminate possible interferences due to ascorbic acid and anthocyanins, the content of these reducing molecules in all fruits was also carried out.

Similitude and differences were discussed on the light of the chemical characteristics of the assay reactions.

Table 1: Selected fruits and their abbreviation.

Scientific name	Common name	Abbreviation
Persea americana	Avocado	Av
Annona muricata	Graviola	Gr
Solanum paniculatum	Jurubeba	Ju
Mangifera indica	Mango Haden	MH
Mangifera indica	Mango Palmer	MP
Mangifera indica	Mango Tommy Atkins	MT
Caryocar brasiliense	Pequi	Pe
Hylocereus undatus	Pitaya	Pi
Spondias purpurea	Purple mombin (siriguela)	Si
Averrhoa carambola	Starfruit (carambola)	St
Tamarindus indica	Tamarind	Ta
Spondias tuberosa	Umbu	Um
Rubus ulmifolius	Blackberry	Ba
Vaccinium cyanococcus	Blueberry	Bu
Rubus idæus	Raspberry	Ra
Ribes rubrum	Redcurrant	Re
Fragaria	Strawberry	Sw

2. Materials and Methods

2.1. Chemicals. All chemicals, of the highest available quality, were obtained from Sigma Chemical Co. (St. Louis, USA); ABIP (2,2′-azobis[2′-(2-imidazolin-2-yl)propane] dihydrochloride) was obtained from Wako Chemicals (Germany). The aqueous solutions were prepared with quality milli-Q water. Each experiment was in triplicate.

2.2. UV-VIS and Electrochemical Measurements. Spectrophotometric measurements were recorded on a UV-VIS Shimadzu UV-1800 instrument equipped with a temperature controlled quartz cell. The measures of oxygen consumption were performed with a potentiostat Amel 559, equipped with an oxygen microelectrode (MI-730, Microelectrodes).

2.3. Fruits and Sample Pretreatments. Table 1 reports common and scientific names of all studied fruits. Mango, avocado, carambola, and pitaya were from Sao Paulo state, while jurubeba, umbu, graviola, pequi, siriguela, and tamarind were from tropical Brazil; soft fruits were from Italy. After cleaning with distilled water, edible fruits portions were grated and centrifuged by a Krups centrifuge under nitrogen flux to avoid the oxidation of the natural components, and the juice was immediately analysed.

2.4. Inhibition of Lipid Peroxidation (ILP). The antioxidant activity of fruits to prevent linoleic acid (LA) peroxidation was determined in sodium dodecyl sulfate (SDS) micelles. As previously reported [27], the fruit's antioxidant capacity was calculated as the juice concentration (ppm) halves the rate of oxygen consumption due to the peroxidation process, and it is expressed as inhibitory concentration IC_{50}.

2.5. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay. This method is based on the capacity of an antioxidant to scavenge the stable free radical DPPH [28]. The procedure is reported in Stevanato et al. [18]; the results are expressed as catechin equivalent concentration (CE).

2.6. Folin-Ciocalteu Assay and Total Phenolics Content (TPC) by Enzymatic Method. The Folin-Ciocalteu assay and the Total Phenolic Content were determined spectrophotometrically, according to the procedures previously reported [18], and the results were expressed as catechin equivalent (CE).

2.7. Total Hydroxycinnamic Acid Content (HCA). Hydroxycinnamic acid content was determined according to Zaporozhets et al. [29]. The complex of hydroxycinnamic acids with aluminium (III) was measured at 365 nm, and caffeic acid was used as a standard; the results were expressed as milligrams/liter of caffeic acid equivalents.

2.8. Total Anthocyanin Content (TAC). The TAC was determined according the pH-differential method [30]. Absorbance at 510 and 700 nm of juice buffered at pH 4.5 e 1.0 was calculated. The anthocyanin concentration was expressed as milligrams/liter of cyanidin-3-glucoside equivalents.

Table 2: Results obtained by ILP, DPPH, TPC, Folin, HCA, and TAA assays of selected fruits.

Fruit	Abbreviation	ILP IC_{50} (ppm)	DPPH CE (mM)	TPC CE (mM)	Folin CE (mM)	HCA (mg/L)	TAA (mM)
Avocado	Av	240 ± 20	0.1 ± 0.01	1.06 ± 0.05	1.99 ± 0.04	56 ± 6	3.1 ± 0.2
Graviola	Gr	87 ± 9	2.4 ± 0.6	3.7 ± 0.5	8.6 ± 0.4	42 ± 3	4.7 ± 0.2
Jurubeba	Ju	60 ± 8	0.9 ± 0.3	7.8 ± 0.5	36 ± 2	3242 ± 20	3.4 ± 0.2
Mango Haden	MH	200 ± 20	0.31 ± 0.05	0.75 ± 0.09	5.7 ± 0.2	184 ± 12	8.6 ± 0.8
Mango Palmer	MP	240 ± 20	0.89 ± 0.01	1.21 ± 0.05	4.5 ± 0.3	80 ± 9	5 ± 1
Mango Tommy Atkins	MT	300 ± 20	0.50 ± 0.07	0.15 ± 0.03	1.4 ± 0.1	90 ± 10	3.7 ± 0.6
Pequi	Pe	500 ± 50	0.1 ± 0.01	0.5 ± 0.1	7.9 ± 0.2	66 ± 7	2.4 ± 0.3
Pitaya	Pi	1000 ± 100	0.1 ± 0.01	1.6 ± 0.2	2.1 ± 0.2	152 ± 12	2.6 ± 0.2
Siriguela	Si	44 ± 4	8 ± 1	3.2 ± 0.1	34 ± 5	264 ± 23	4.7 ± 0.3
Carambola	St	70 ± 7	2.5 ± 0.1	5.4 ± 0.4	10.5 ± 0.1	164 ± 14	4.2 ± 0.3
Tamarind	Ta	100 ± 20	2.4 ± 0.3	2.9 ± 0.1	18.5 ± 0.8	168 ± 15	7 ± 1
Umbu	Um	500 ± 30	0.67 ± 0.05	1.4 ± 0.2	4.2 ± 0.1	46. ± 3	1.5 ± 0.2
Blackberry	Ba	109 ± 6	3.0 ± 0.2	2.66 ± 0.06	8.4 ± 0.1	203 ± 19	4.4 ± 0.6
Blueberry	Bu	41 ± 7	3.4 ± 0.1	2.8 ± 0.1	7.8 ± 0.5	350 ± 25	3.0 ± 0.1
Raspberry	Ra	77 ± 9	4.2 ± 0.1	2.7 ± 0.1	21 ± 1	101 ± 9	3.7 ± 0.3
Redcurrant	Re	56 ± 2	3.4 ± 0.4	3.9 ± 0.4	10.0 ± 0.1	180 ± 12	4 ± 0.4
Strawberry	Sw	38 ± 4	4.1 ± 0.3	3.04 ± 0.09	10 ± 3	190 ± 13	6 ± 1

2.9. Total Ascorbic Acid (TAA). The TAA is assayed as previously described [31] with minor modifications. A 20 mM oxalic acid solution containing the sample, 0.186 mM 2,6-dichlorophenol-indophenol (DCFI), 10 mM dinitrophenylhydrazine (DNPH), and 13 mM thiourea were incubated in a boiling water bath for 15 minutes. Once cooled, an equal volume of 85% sulfuric acid was added to the solution, and the absorbance at 520 nm was measured 15 minutes later. The same procedure was repeated without the sample, and the blank value was subtracted from the absorbance of the sample.

In Table 2, where the results obtained by applying ILP, Folin, DPPH, and TPC enzymatic methods are reported, it appears that jurubeba and siriguela show very low IC_{50} values (i.e., high antioxidant activity) in the range of those found for the more active Italian soft fruits (blueberry, redcurrant, and raspberry). For the same fruits, DPPH, TPC, and Folin assays give very high values of CE, if compared with the average of other fruits, indicating an univocal high antioxidant activity of these two fruits.

On the basis of their IC_{50} values, the investigated Brazilian tropical fruits can be roughly divided into three groups characterized approximately by good, medium, and poor antioxidant properties, respectively (Figure 1): (1) fruits with $\log(IC_{50}) \leq 2$ ($IC_{50} \leq 100$ ppm): graviola, jurubeba, siriguela, carambola, and tamarind; (2) fruits with $2 < \log(IC_{50}) \leq 2.5$ (IC_{50} ranging from 100 to 316 ppm): avocado and mango; (3) fruits with $\log(IC_{50}) > 2.5$ ($IC_{50} > 316$ ppm): pequi, umbu, and pitaya.

In Figure 2, correlations between data obtained by ILP expressed as $1/IC_{50}$ and other adopted methods expressed as catechin equivalent amount (CE) are reported.

The comparison of the data obtained by ILP versus DPPH scavenging methods (Figure 2(a)) points out a good correlation ($R = 0.79$); in fact only few points referred to that strawberry, blueberry, jurubeba, and, in less amount, siriguela scatter from the linear relationship.

Analogous graph created for comparison of ILP with enzymatic or the Folin methods (Figures 2(b) and 2(c)) shows less good correlations ($R = 0.60$ and 0.30, resp.), but also in this case strawberry, blueberry, jurubeba, and, in part, siriguela appear to worsen the correlation coefficient.

TAC measurements showed the absence of anthocyanins in analyzed Brazilian fruits, while as regards the hydroxycinnamic acid content, the values of HCA equivalents obtained for the studied fruits and reported in Table 2 show a very high value of HCA_{eq} for jurubeba.

No correlation appears comparing TAA values with the data obtained by the other analytical methods (data are not showed).

3. Discussion

3.1. On the Assay Methods. Several methods are proposed to evaluate the antioxidant activity of molecules or food [26–28, 32–35]. Each assay measures a specific chemical or physicochemical parameter which can be correlated with the complex and in part unknown mechanisms related to ROS injury. It follows that the results obtained are partial and sometime are affected by other variables not strictly correlated to the antioxidant activity. In this work, we chose four different assays which significantly represent the main methods of measuring the antioxidant properties of a substance.

The Folin-Ciocalteu is a very aged and largely used assay, based on the absorbance changes due to the oxidation of any

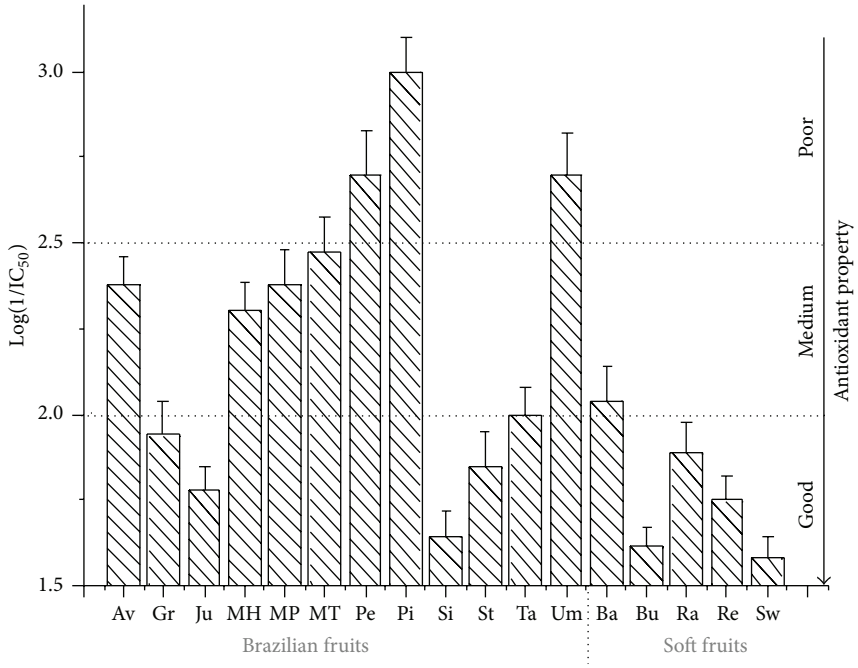

Figure 1: Classification of studied fruits on the base of their logarithm IC_{50} values.

reduced compounds by a phosphomolybdate and phosphotungstate solution. It is a nonspecific method of measuring the reducing capacity of all the components of the sample other than polyphenols, such as ascorbate [18, 36]. In fact, to avoid an overestimated evaluation of the antioxidant capacity, laborious pretreatments of the sample are suggested [37].

TPC enzymatic method, on the contrary, being a measure of the total phenolic content of fruit due to the specificity of peroxidase-catalyzed reaction towards phenolic structures, is an indirect evaluation of the antioxidant power, which actually depends not only on the measured total phenolic content, but also on the chemical structure of each phenolic component [14].

DPPH method is a measure of the electronic transfer from the phenolic structure to the stable free radical DPPH, but this reaction presents the following disadvantages which can underestimate the antioxidant capacity:

(i) it may react slowly or be inert to many antioxidants [38];

(ii) reaction kinetic with antioxidants appears not linear to DPPH concentrations [36];

(iii) reaction of DPPH with some phenolic structures could not go to completion, reaching an equilibrium state, as found for eugenol [36].

4. Results

By a physicochemical point of view, ILP technique appears to better reproduce the *in vivo* action of antioxidant substances against radical-induced lipid peroxidation of unsaturated fatty acids residues of biological membranes, measuring *in vitro* the slowdown, due to an antioxidant, of the oxygen consumption in linoleic acid containing SDS micelles. In this case, the influence due to the different lipophilicity of the antioxidant molecules is taken in account too. Moreover, in this work, only clear juices have been analyzed, and, as a consequence, only water soluble antioxidants have been assessed.

Anyway, in order to be certain of the data reliability and to give a wider outlook of the problems related to the definition of the antioxidant activity of foods, the same samples were studied by the above cited four analytical assays, and the results were compared to put in light possible correlations. In fact, good correlations between results obtained by different assays can guarantee the best evaluation of the antioxidant properties of a sample.

4.1. On the Antioxidant Characteristics of Brazilian Fruits. Siriguela, jurubeba, carambola, graviola, and tamarind show high antioxidant activity, similar to that of soft fruits [25, 26]. This result appears very important considering that for some of these fruits no information in literature is reported, in particular about their antioxidant properties [39]. Moreover, the widespread use for curative actions into local populations of some of these fruits, in particular jurubeba and siriguela, suggests further investigations for their possible nutraceutical properties.

With reference to the scattering from the linear correlation of the data referred to strawberry, blueberry, jurubeba, and siriguela, as it results in all three graphs of Figure 2, plots of correlation of the data obtained by DPPH, Folin, and enzymatic methods are graphed in order to verify if this deviation could be due to a limit of the ILP assay (Figure 3).

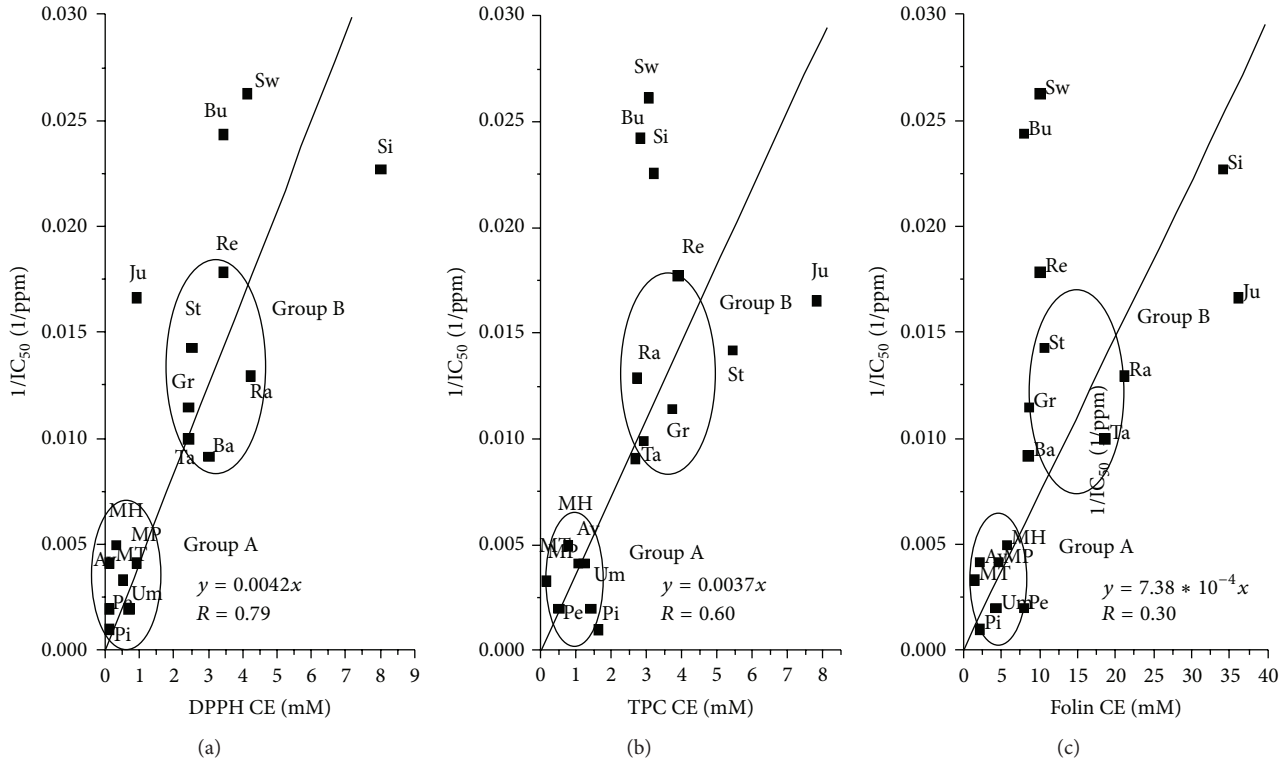

Figure 2: Correlation between ILP and (a) DPPH, (b) enzymatic, and (c) Folin assay.

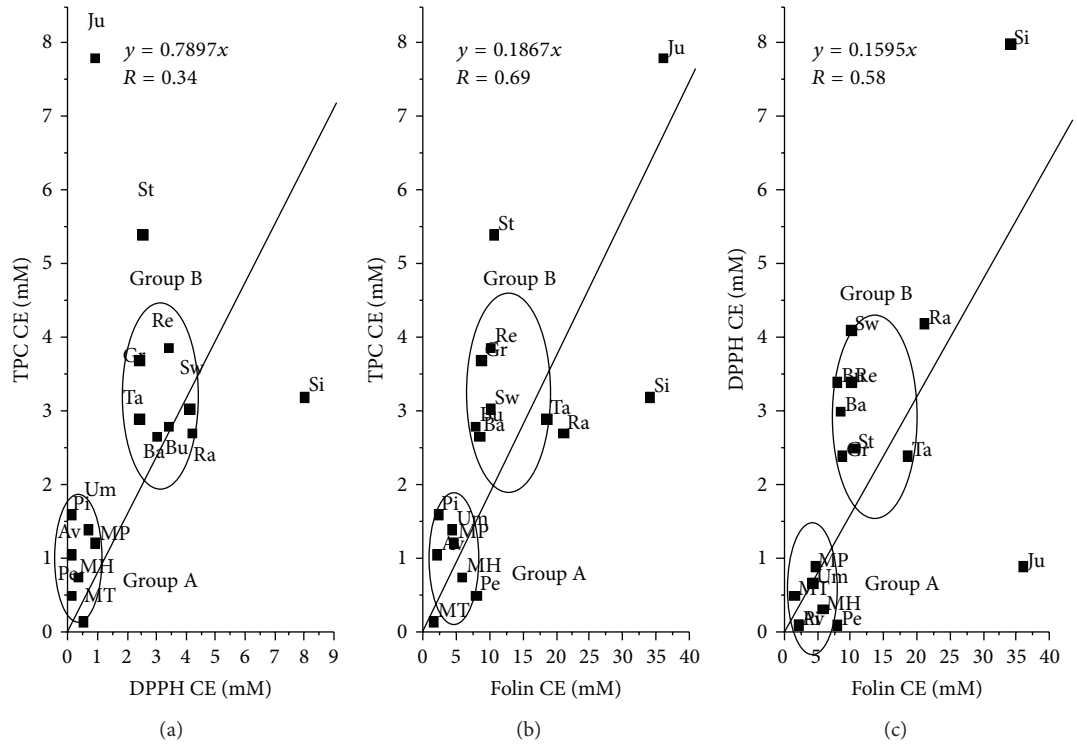

Figure 3: Correlation between (a) enzymatic and DPPH; (b) enzymatic and Folin; (c) DPPH and Folin assays.

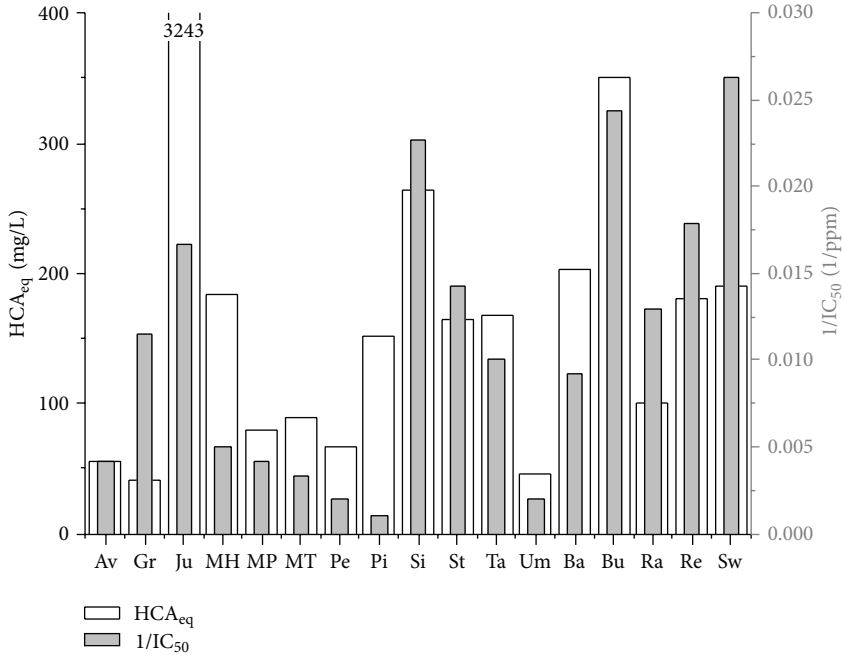

Figure 4: Correlation between ILP and HCA equivalents.

Also in these cases, the data of the above-mentioned fruits appear considerably out of the correlation straight line, indicating that the chemical compounds that are responsible of the antioxidant activity are differently recorded by the different analytical methods.

Jurubeba and siriguela are two striking examples of how different assays may assign different rankings to antioxidant molecules: as it appears in Figure 3(a), while the antioxidant activity of jurubeba is high when evaluated by the enzymatic method and low when evaluated by DPPH, in the case of siriguela the DPPH method assigns it excellent antioxidant properties which are not confirmed by the enzymatic assay. The result of the first case can be due to the high content in jurubeba of polyphenols characterized by a low tendency to undergo monoelectronic transfer to DPPH, as recently verified for different flavonoids [14]. Further investigations to clarify this contrasting behaviour are necessary in any case.

The better correlation results from the comparison of the ILP and DPPH data (Figure 2(a)). In fact, both the analytical methods are based on the redox potentials of the monoelectronic transfer, and they appear in some way as a direct measure of the radicals stopping power [28, 39] of the antioxidant substances in the fruit. Moreover, the joint data obtained by IC_{50} and DPPH experiments are particularly efficient for separating poor antioxidants from good ones: IC_{50} values that are lower than 100 ppm and/or CE values that are higher than 2 mM could be assumed as a reasonable rule for discriminating very good antioxidants.

Even if there is a bad correlation between DPPH and enzymatic data (Figure 3(a)), most of the fruit can be roughly separated in two groups (A and B) with different degrees of antioxidant activity, suggesting the hypothesis that fruit of the same group could have quite similar compositions of antioxidant constituents or molecules which react in similar way to the analytical methods.

Anthocyanins are not contained in examined Brazilian fruits, while hydroxycinnamic acids are detected; their correlation with ILP is practically absent, as shown in Figure 4. For this reason, antioxidant property must depend on other parameters.

Table 2 indicates that, in general, Brazilian fruits have ascorbic acid content comparable to that of soft fruits: among them two varieties of mango and tamarind have meaningfully high TAA content, and umbu have the lowest one.

No evident relationship between the antioxidant activity of fruit and the content of ascorbic acid is observed: siriguela and jurubeba have the highest antioxidant activity, but they exhibit lower values of vitamin C than mango, which is not a good antioxidant instead (Table 2). It follows that antioxidant activity of the majority of fruits is due to compounds different from vitamin C, like polyphenols, mainly flavonoids, according to results reported for other species of fruit [11, 22].

5. Conclusion

Brazilian fruits were used as arbitrary alimentary products to compare four different assays normally utilised to determine antioxidant activity of food.

The better correlation was found between the inhibition of lipid peroxidation and DPPH method. Both these assays are based on monoelectronic transfer, and, in our opinion, they mime, more than others, the efficacy of an antioxidant compound to prevent oxidative damage on cell membrane, despite all the limitations of the DPPH assay above reported

and taking into account the laboriousness of the ILP method. From data obtained by these two methods, siriguela and jurubeba show the higher antioxidant activity.

The antioxidant activity of the majority of the studied fruit is due to compounds different from vitamin C, like flavonoids, because no evident relationship between the antioxidant activity of fruit and the content of ascorbic acid was observed.

Conflict of Interests

This work is free from any conflict of interests.

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Research Article

Evaluation of *In Vivo* Wound Healing Activity of *Bacopa monniera* on Different Wound Model in Rats

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Wound healing effects of 50% ethanol extract of dried whole plant of *Bacopa monniera* (BME) was studied on wound models in rats. BME (25 mg/kg) was administered orally, once daily for 10 days (incision and dead space wound models) or for 21 days or more (excision wound model) in rats. BME was studied for its *in vitro* antimicrobial and *in vivo* wound breaking strength, WBS (incision model), rate of contraction, period of epithelization, histology of skin (excision model), granulation tissue free radicals (nitric oxide and lipid peroxidation), antioxidants (catalase, superoxide dismutase, and reduced glutathione), acute inflammatory marker (myeloperoxidase), connective tissue markers (hydroxyproline, hexosamine, and hexuronic acid), and deep connective tissue histology (dead space wound). BME showed antimicrobial activity against skin pathogens, enhanced WBS, rate of contraction, skin collagen tissue formation, and early epithelization period with low scar area indicating enhanced healing. Healing effect was further substantiated by decreased free radicals and myeloperoxidase and enhanced antioxidants and connective tissue markers with histological evidence of more collagen formation in skin and deeper connective tissues. BME decreased myeloperoxidase and free radical generated tissue damage, promoting antioxidant status, faster collagen deposition, other connective tissue constituent formation, and antibacterial activity.

1. Introduction

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical stability and disturbed functional status of the skin. Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation, and migration of different cell types [1]. The inflammation stage begins immediately after injury, first with vasoconstriction that favors homeostasis and releases inflammation mediators. The proliferative phase is characterized by granulation tissue proliferation formed mainly by fibroblast and the angiogenesis process. The remodeling stage is characterized by reformulations and improvement in the components of the collagen fibre that increases the tensile strength [2]. Factors that contribute to causation and perpetuation of the chronicity of wounds include repeated trauma, poor perfusion or oxygenation, and excessive inflammation [3]. Imbalance in free radical generations and antioxidants has been observed to induce oxidative stress and tissue damage and delayed wound healing. Therefore, elimination of ROS could be an important strategy in healing chronic wounds [4].

Bacopa monniera (BM, Scrophulariaceae) called as water hyssop is a prostrate herb, commonly found in wet or marshy habitats and along the stream and river margins throughout India. The Charaka Samhita considers BM (Synonyms: *Herpestis monniera*) as medhya rasayana and Ayurvedic texts advocate the use of BM in ascites, enlarged spleen, indigestion, inflammation and leprosy, and so forth and, as a result, researchers have evaluated its sedative and tranquillizing, cognition, antidepressant and antianxiety, antiepileptic, antioxidant and adaptogenic, antiulcer, and anti-*Helicobacter* properties [5–13]. Recently, alcoholic extract of *Bacopa monniera* and its isolated constituent Bacoside-A were screened for wound healing activity by excision, incision, and dead space wound on Swiss albino rats and were found to enhance

wound healing in terms of increase in tensile strength, wound epithelization, and connective tissue formation [14].

The present study was, therefore, undertaken to do an in-depth study on the wound healing activities of 50% ethanol extract of whole plant of *Bacopa monniera* in incision, excision, and dead space wound models in rats when given by oral route.

2. Materials and Methods

2.1. Animals. Inbred Charles-Foster albino rats (160–180 g) of either sex were obtained from the central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi. They were kept in the departmental animal house at 26 ± 20°C and relative humidity 44–56%, light and dark cycles of 10 and 14 h, respectively, for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Pashu Aahar Vihar, Ramnagar, Varanasi) and water *ad libitum*. "Principles of laboratory animal care" (NIH publication no. 82-23, revised 1985) guidelines were followed. Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work (Notification no. Dean/2010-11/275 dated 13.10.2010).

2.2. Plant Material and Preparation of Extract. The whole plant of *Bacopa monniera* (BM) (Ayurvedic Gardens, Banaras Hindu University) was collected during April–June and identified with the standard sample preserved in the department of Dravyaguna, Institute of Medical Sciences, Banaras Hindu University, Varanasi. 50% ethanolic extract of BM (BME) was prepared by adding 500 g of dried, crushed, and powdered whole plant of BM in 1000 mL of 50% ethanol in a round bottom flask and was kept at room temperature for 3 days in shade. The extract was filtered and the previous procedure was repeated twice. The extract filtrate so obtained was pooled and evaporated on water bath till it dried. The yield of BME was about 28.16% (w/w).

2.3. Drug and Chemicals. Vitamin E (Merck Ltd., Mumbai, India) and all the other chemicals and reagents were used of analytical grade.

2.4. Dose Selection and Treatment Protocol. A preliminary dose-response effect using BME was first undertaken to study the wound breaking strength, in incision wound model, in rat. Graded doses of BME 12.5, 25, and 50 mg/kg were administered once daily orally for 10 days in rats following induction of incision wound. The sutures were removed on 7th day of experiment and wound breaking strength (WBS) was measured on 10th postwounding day. The result of the dose response study in incision wound model indicated that 25 mg/kg of BME had the optimal effect. Therefore, dose of 25 mg/kg of BME was chosen for further study on various physical, biochemical, and histopathological parameters of wound healing in rat dead space wound models. BME (25 mg/kg) and the standard drug Vitamin E (VTE; 200 mg/kg), suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water, were given orally once daily from day 1, 4 hours after the induction of excision and dead space wounds. The animals received CMC/BME/VTE orally with the help of an orogastric tube in the volume of 1 mL/100 g body weight for 10 days for dead space wound and incision wound studies and 20 days or till the period of complete epithelization for excision wound study.

2.5. Wound Healing Studies

2.5.1. Incision Wound Model. Two parallel six cm paravertebral incisions were made through the full thickness of the skin, 1 cm lateral to the midline of vertebral column after giving anaesthesia [15]. Wounds were closed with interrupted sutures, 1 cm apart, with surgical suture. The sutures were removed on the 7th postwounding day. Wound breaking strength (WBS) was measured on the 10th postwounding day in anaesthetized rats. A line was drawn on either side of the incision line 3 mm away from the wound. Two Allis forceps were firmly applied on to the line facing each other. One of the forceps was fixed, while the other was connected to a freely suspended lightweight polypropylene graduated container through a string run over to a pulley. Standard weights were put slowly and steadily into the container. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges. As and when the wound just opened up, the weight was stopped and noted.

2.5.2. Excision Wound Model. Rats were anesthetized with ketamine (30 mg/kg, ip) and an area of about ≈500 mm^2 was marked on the back of the rat by a standard ring. Full thickness of the marked skin was then cut carefully. Wounds were traced on 1 mm^2 graph paper on the day of wounding and subsequently at a gap period of 4 days till 12th day, then on the alternate days until healing was complete. Changes in wound area were measured regularly and the rate of wound contraction calculated as given in the formula below. Significance in wound healing of the test groups is derived by comparing healed wound area on respective days with healed wound area of control group. The period of epithelization, that is, day of fall of eschar and the scar area, was also noted down [15]:

$$\%\ \text{wound contraction} = \left[\frac{\text{Healed area}^{\circ}}{\text{Total wound area}}\right] \times 100, \quad (1)$$

$$\left(^{\circ}\text{Healed area} = \text{original wound area} - \text{present wound area}\right).$$

2.5.3. Dead Space Wound Model. Rats were anesthetized with ketamine and 1 cm incision was made on dorsolumbar part of the back. Two polypropylene tubes (0.5 × 2.5 cm^2 each) were placed in the dead space of lumbar region of rat on each side, and wounds were closed with a suture material. On the 10th postwounding day, the animals were sacrificed and granulation tissue formed on and around the implanted tubes was carefully dissected out, weighed, and processed for the estimation of free radicals, antioxidants, and collagen tissue parameters [15].

2.6. Estimation of Granulation Tissue Free Radical and Antioxidant. Antioxidants—superoxide dismutase, SOD [16]; catalase, CAT [17] and reduced glutathione, GSH [18]; free radicals—lipid peroxidation, LPO [19] and nitric oxide, NO [20] and acute inflammatory marker, myeloperoxidase (MPO) [21], and protein [22] were estimated in wet granulation tissue homogenates. Briefly, the wet granulation tissues were homogenized in a glass Teflon homogenizer (10% w/v) at 4°C in Phosphate buffered saline (PBS, pH 7) used for the estimation of protein, free radicals, and antioxidants. The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitro blue tetrazolium formazan. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of nitro blue tetrazolium reduction in one minute. CAT measurement was done based on the ability of catalase to oxidize hydrogen peroxide. One unit (U) of catalase is the enzyme, which decomposes one mM of H_2O_2/min at 25°C. GSH activity in the homogenate was estimated by the ability of GSH to reduce DTNB within 5 min of its addition against blank. LPO levels were estimated in terms of malondialdehyde (MDA) released during lipid peroxidation Nitrites and nitrates are formed as end products of reactive nitrogen products during NO formation which are measured by using Griess reagent.

For myeloperoxidase (MPO) estimation, granulation tissue (5% w/v) was homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma-Aldrich, Co., St. Louis, MO, USA) with 50 mM potassium phosphate buffer (pH 6). The previous homogenate was freeze-thawed three times, sonicated for 10 seconds, and then centrifuged at 14000 ×g for 45 minutes at 4°C and the resulting supernatant was used for estimation of MPO. A unit of MPO activity is defined as that converting 1 μmol of H_2O_2 to water in 1 min at 25°C.

2.7. Estimation of Connective Tissue Parameters. Approximately 250 mg of wet tissue was dried at 50°C for 24 h. It was weighed and kept in glass stoppered test tubes. To each tube containing 40 mg of the dried granulation tissue, 1 mL of 6 N HCl was added. The tubes were then kept on boiling water bath for 24 h (12 h each day for two days) for hydrolysis. The hydrolysate was then cooled and excess of acid was neutralized by 10 N NaOH using phenolphthalein as indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/mL with distilled water. The final hydrolysate was used for the estimation of hydroxyproline, hexosamine, and hexuronic acid following the standard curve prepared using the proper substrate.

2.7.1. Hydroxyproline (HPR). To the each tube, 0.3 mL each of hydrolysate, 2.5 N NaOH, 0.01 M $CuSO_4$, and 6% H_2O_2 were added. Tubes were shaken vigorously and placed immediately in water bath at 80°C. After 15 minutes, tubes were removed and cooled for 5 minutes in cold water. 0.6 mL of freshly prepared 5% solution of paradimethyl amino-benzaldehyde in n-Propanol and 1.2 mL of 3 N H_2SO_4 was added. The test tubes were once again placed in a hot water bath at 75°C for 15 minutes and then cooled for 5 minutes under running stream of water. Color intensity was measured at 540 nm against the blank. Hydroxyproline content in the tissue was estimated as per standard curve prepared with standard 4-Hydroxy-L-proline (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 75 to 900 μg/0.3 mL using 3 mg/mL working solution [23].

2.7.2. Hexosamine (HXA). 0.05 mL of hydrolyzed fraction was diluted to 0.5 mL with distilled water. To this was added 0.5 mL of acetyl acetone reagent and heated in boiling water bath for 20 min then cooled under tap water. To this 1.5 mL of 95% alcohol was added, followed by an addition of 0.5 mL of Ehrlich's reagent. The reaction was allowed for 30 minutes to complete. Color intensity was measured at 530 nm against the blank. Hexosamine content of the samples was determined from the standard curve prepared with D (+) glucosamine hydrochloride (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 5 to 50 μg/0.5 mL using 100 μg/mL working solution [24].

2.7.3. Hexuronic Acid (HUA). 2.5 mL of 0.025 M Borax in concentrated sulphuric acid is placed in stoppered tubes fixed in a rack and cooled to 4°C. 0.125 mL of hydrolysate was diluted 0.5 mL by adding distilled water. Now, this 0.5 mL of hydrolysate is layered carefully on Borax-sulphuric acid mixture kept in rack at 4°C. The tubes were closed with glass stoppers and then shaken, first slowly then vigorously, with constant cooling by placing tubes in ice container. The tubes were then heated for 10 min in a vigorously boiling water bath and cooled to room temperature. Thereafter, 0.1 mL of 0.125% carbazole reagent in absolute alcohol was then added to each tube, shaken, again heated in the boiling water bath for further 15 min, and then cooled to room temperature. Color intensity was measured at 530 nm against the blank. Hexuronic acid content of the samples was determined from the standard curve prepared with D (+) Glucurono-6, 3-lactone (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 5 to 40 μg/0.5 mL using 100 μg/mL working solution [25].

2.8. Histopathology. The cross-sectional full-thickness skin specimens and deep granulation tissues from the implanted tube were collected on the 10th day of the experiment for the histopathological alterations. Samples were fixed in 10% buffered formalin, processed, blocked with paraffin, then sectioned into 5 μm sections, and stained with hematoxylin and eosin.

2.9. Antimicrobial Susceptibility and Minimum Inhibitory Concentration (MIC). *In vitro* antibacterial susceptibility test of BME was done using serial concentrations of 50, 100, 150, and 200 mg/mL following the approved standards of the National Committee for Clinical Laboratory Standards (NCCL) [26] against common skin bacteria *Staphylococcus aureus* (ATCC 25323), *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* obtained from the American Type Culture Collection (ATCC) and clinical strain preserved at Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India, following the disk diffusion method

[27] while minimum inhibitory concentration (MIC) was performed by microdilution method [28]. Briefly, 24 h old culture of selected microbes was adjusted to 0.5 McFarland standard in sterile normal saline to achieve concentration of ~10^7 (colony forming units) CFU/mL. Standard antibiotics used as positive control. Dimethylsulfoxide (DMSO) was used as negative control. MIC was determined by microbroth dilution method. Specifically 0.1 mL of standardized inoculums of bacteria (1-2 × 10^7 CFU/mL) was added in each well of microtiter plate which was incubated aerobically at 37°C for bacterial growth for 18–24 h. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control was regarded as MIC.

2.10. Statistical Analysis. Statistical comparison was performed using either unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons" instead of "for multiple comparisons verses control group was done by Dunnett's test. All statistical analysis was performed using SPSS statistical version 16.0 software package (SPSS Inc., USA).

3. Results

3.1. Incision Wound Model. Control rats showed WBS as 283.3 ± 18.6 g on 10th postwound day. BME 12.5, 25, 50 mg/kg treated rats showed WBS as 353.3 ± 19.3 g, 373.3 ± 13.8 g, 375.0 ± 14.8 g ($P < 0.05$ to $P < 0.01$), respectively, while vitamin E (VTE, 200 mg/kg) treated rats showed WBS as 405.0 ± 21.1 g ($P < 0.01$).

3.2. Excision Wound Model. Rate of wound contraction in control rats was 21.6% to 68.3% from day 4 to day 12 and 80.6% to 98.1% from day 14 to day 20, while complete epithelization and healing were observed on day 24. The average number of days that took for the shedding of eschar without leaving any residual raw wound in these rats was 12.7 days and mean of scar area after completing healing was 99.8 mm^2. The percent rate of wound contraction in rats, treated orally with BME (25 mg/kg), was from 32.2% on day 4 to 85.4% on day 12 and 92.1% to 100% from day 14 to day 20, respectively, while VTE treated rats showed increase in wound contraction from 32.4% on day 4 to 87.6% on day 12 and 92.2% to 100% from day 14 to day 20, respectively. The mean epithelization period and scar area observed with BME were 10.3 days and 75.2 mm^2 while the mean epithelization period and scar area observed with VTE were 10 days and 74.3 mm^2, respectively. BME treated rats, thus, showed faster healing which was comparable with VTE treated group (Table 1 & Figure 1).

Histology of excision biopsy of skin wound at day 10 showed healed skin structures with normal epithelization, restoration of adnexa and fibrosis within the dermis in BME and VTE treated groups while the control group lag behind treated group in formation of the amount of ground substance in the granulation tissue, as observed in tissue sections (Figure 2).

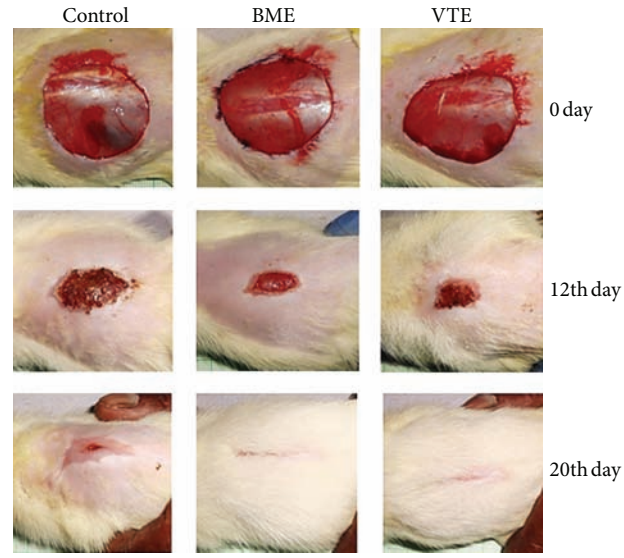


Figure 1: Photographic representation of contraction rate showing percent wound contraction area on different postexcision days of control, BME (25 mg/kg), and VTE (200 mg/kg) treated rats.

3.3. Dead Space Wound Model. BME caused an increase in wet weight mg per 100 g body weight and protein mg/g granulation tissue by 17.6% ($P < 0.05$) and 18.7% ($P < 0.05$), respectively, compared with control group. BME effect was comparable with that of VTE on the previous parameters (Table 2).

3.4. Wet Granulation Tissue Antioxidants, Free Radicals, and Myeloperoxidase. BME showed significant increase in the level of antioxidants, GSH, SOD, and CAT while free radicals, LPO and NO, and acute inflammatory marker, MPO were decreased. The results with BME were comparable with VTE on the previous soft-tissue parameters (Table 2).

3.5. Dry Connective Tissue HPR, HXA, and HUA. Dry weight of granulation tissue and protein content were increased by 19.4% and 18.1% ($P < 0.05$), respectively, in BME treated groups compared with control group. HPR, HXA, and HUA were significantly increased in BME treated group by 59.8%, 59.1%, and 153%, respectively, compared to control group. The results with BME were comparable with VTE on the previous connective tissue parameters (Table 3).

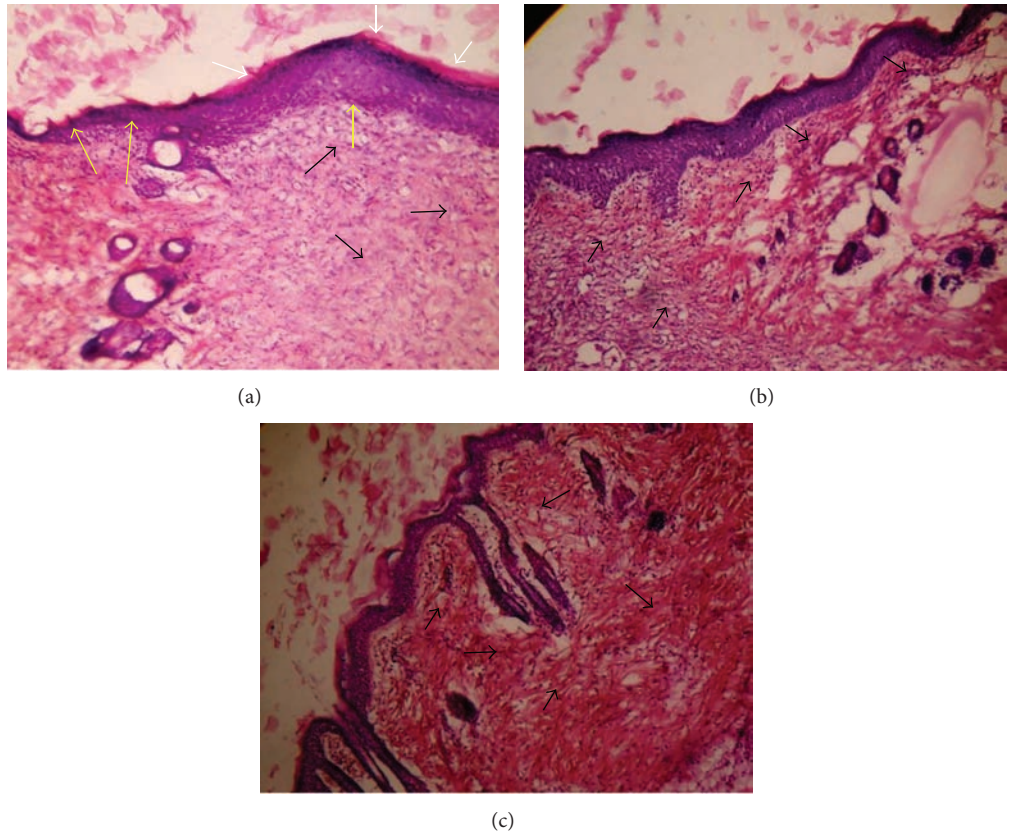
Histology of granulation tissue of deeper structure of control rat showed mononuclear inflammatory cells, scattered fibroblasts (minimal fibrosis), and few proliferating vasculature in granulation tissue, while the granulation tissue of rats treated with BME and VTE showed abundance of eosinophilic collagen tissue and neovascularisation with few inflammatory cells indicative of healing by fibrosis (Figure 3).

3.6. Antimicrobial Susceptibility and MIC. BME showed positive susceptibility test against common skin bacteria *Staphylococcus aureus* (ATCC 25323), *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*, showing zone of inhibition ≥10 mm, at 200 mg/mL. BME had least minimum inhibitory

Table 1: Effect of BME and VTE on wound contraction, epithelization period, and scar area in excision wound.

Oral treatment (mg/kg, od)	Wound area in mm^2/rat (% contraction)									Epithelization period (days)	Scar area (mm^2)
	0 day	4th day	8th day	12th day	14th day	16th day	18th day	20th day	22nd day		
Control 0.5% CMC	532.8 ± 6.93 (0.00)	417.7 ± 6.77 (21.6 ± 1.0)	322.3 ± 10.8 (39.5 ± 1.73)	168.5 ± 10.1 (68.3 ± 2.03)	103.3 ± 5.79 (80.6 ± 1.16)	81.2 ± 2.36 (84.8 ± 0.48)	36.2 ± 1.25 (93.2 ± 0.20)	10.3 ± 0.67 (98.1 ± 0.15)	3.17 ± 0.75 (99.4 ± 0.15)	12.7 ± 0.67	99.8 ± 4.92
BME 25	530.3 ± 7.15 (0.00)	359.2 ± 12.2[b] (32.2 ± 2.71)	217.8 ± 20.8[b] (59.0 ± 3.96)	77.2 ± 5.21[c] (85.4 ± 1.08)	42.0 ± 3.86[c] (92.1 ± 0.72)	22.3 ± 2.33[c] (95.8 ± 0.46)	4.60 ± 0.56[c] (99.0 ± 0.09)	0.0 ± 0.0[c] (100.0)	0.0 ± 0.0[c] (100.0)	10.3 ± 0.49[a]	75.2 ± 4.04[b]
VTE 200	550.0 ± 7.02 (0.00)	371.7 ± 12.1[b] (32.4 ± 2.03)	229.8 ± 14.2[c] (58.3 ± 2.48)	68.3 ± 2.55[c] (87.6 ± 0.49)	42.8 ± 6.13[c] (92.2 ± 1.12)	19.7 ± 3.02[c] (96.4 ± 0.55)	3.83 ± 0.98[c] (99.3 ± 0.17)	0.0 ± 0.0[c] (100.0)	0.0 ± 0.0[c] (100.0)	10.0 ± 0.58[a]	74.3 ± 3.98[b]

Values are mean ± SEM (Percent) of 6 rats in each group. [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$ compared to respective day control group (statistical analysis was done by one-way analysis of variance followed by Dunnett's test for multiple comparisons).



(a) (b) (c)

Figure 2: Histopathology of skin at day 10 stained with H&E (100x). (a) Skin of control rat showing ulceration and edema showed by white arrow, early epithelization showed by yellow arrow, and granulation tissue and abundance of mononuclear inflammatory cells showed by black arrow. (b) BME treated rats showing large amount of granulation tissue by black arrow, small number of mononuclear inflammatory cells, and restoration of adnexa and extensive fibrosis. (c) VTE treated rats showing healed skin structures with well-formed, near to normal epidermis, restoration of adnexa, and extensive fibrosis and collagen tissue within the dermis.

Table 2: Effect of BME and VTE on wet granulation tissue weight, protein, free radicals (LPO and NO), antioxidants (GSH, SOD, and CAT), and myeloperoxidase (MPO).

Oral treatment (mg/kg, od × 10 day)	Wet tissue mg/100 g bw	Protein mg/g tissue	Antioxidants			Free radicals		Myeloperoxidase
			GSH nM/mg protein	SOD IU/mg protein	CAT mU/mg protein	LPO nM/mg protein	NO nM/mg protein	MPO mU/mg protein
Control 0.5% CMC	359.3 ± 18.3	48.6 ± 2.75	20.7 ± 1.17	0.41 ± 0.07	42.9 ± 1.84	6.33 ± 0.50	36.8 ± 4.53	24.1 ± 0.55
BME 25	422.7 ± 18.1[a]	57.7 ± 2.05[a]	24.6 ± 1.11[a]	0.72 ± 0.02[b]	151.6 ± 0.68[c]	3.24 ± 0.26[c]	15.3 ± 0.87[c]	19.3 ± 0.29[c]
VTE 200	461.5 ± 11.5[b]	59.1 ± 3.40[a]	24.5 ± 0.88[a]	0.79 ± 0.04[c]	210.4 ± 0.84[c]	1.78 ± 0.15[c]	15.2 ± 1.53[b]	14.6 ± 0.37[c]

Values are mean ± SEM of 6 rats in each group. [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$ compared to respective control group (statistical analysis was done by one-way analysis of variance followed by Dunnett's test for multiple comparisons).

concentration (MIC) of 0.39 mg/mL against *Staphylococcus aureus* (ATCC 25323) and 3.125 mg/mL against *Pseudomonas aeruginosa*, whereas MIC of BME against other organisms ranged from 6.25 to 25.0 mg/mL.

4. Discussion

Wound represents a major health problem, both in terms of morbidity and mortality. The processes involved in wound healing are epithelization, contraction, and connective tissue deposition. The healing process depends, to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation [15]. In the tissue repair process, inflammatory cells promote the migration and proliferation of endothelial cells, leading to neovascularisation of connective tissue cells which synthesize extracellular matrices including collagen, and of keratinocytes resulting to reepithelialization of the wounded tissue [29]. Inflammation, collagen maturation, and scar formation are some of the

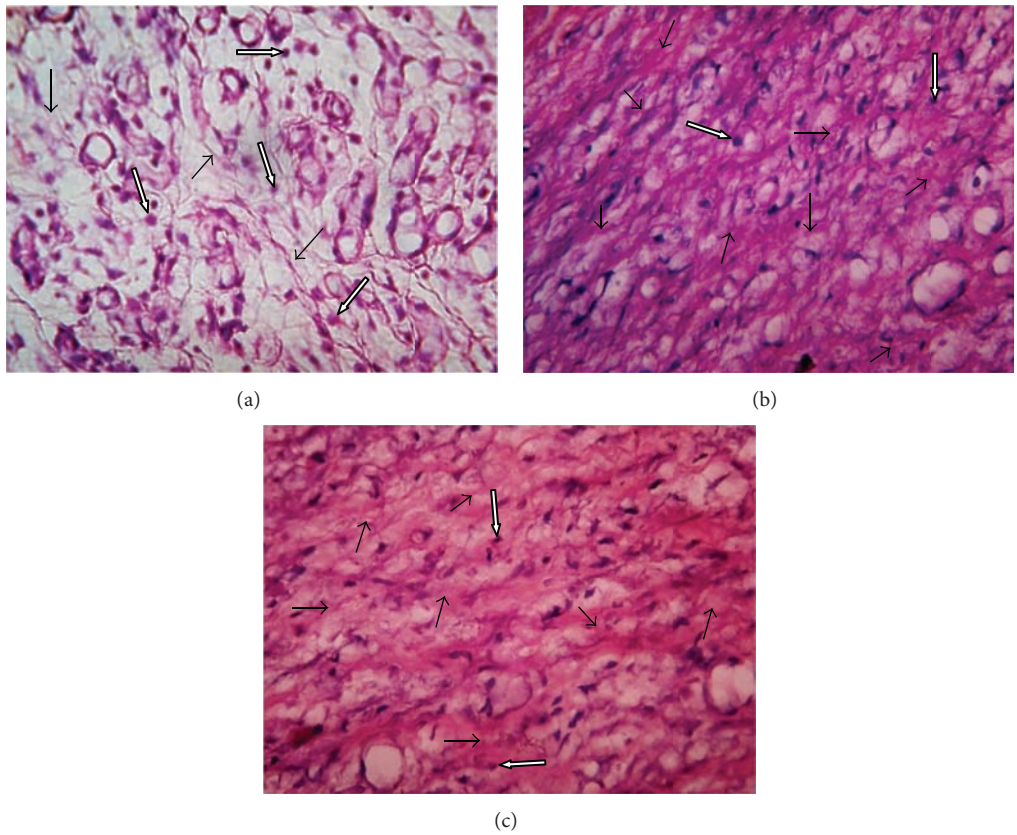


Figure 3: Histopathology of granulation tissue at day 10 stained with H&E (100x). (a) Granulation tissue of control rat showed mononuclear inflammatory cells by white arrow, scattered abundance of eosinophilic fibroblasts showed by black arrow. (b) BME treated showing large number of collagen tissue (fibrosis) and neovascularisation with minimal inflammatory cells. (c) VTE treated showing near to normal features, collagen tissue (fibrosis), and neovascularisation.

many phases of wound healing, which run concurrently but independent of each other.

In incision wound study, BME showed an increase in breaking strength which may be due to the increase in collagen concentration and stabilization of the fibres [30]. The collagen molecules synthesized are laid down at the wound site and become cross-linked to form fibres. Wound strength is acquired from both remodeling of collagen and the formation of stable intra- and intermolecular crosslinks. BME showed greater breaking strength which may be due to increased collagen synthesis as found in the dead space wound study.

In excision wound, BME showed faster healing compared with control group. Further, excision biopsy of skin wound at day 10 showed healed skin structures with normal epithelization, restoration of adnexa and fibrosis within the dermis in BME and VTE treated groups, while the control group lags behind treated group in formation of the amount of ground substance in the granulation tissue. The faster wound contraction by BME may be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes, and may increase the gap junctional intracellular communication in fibroblasts, and induces a more rapid maturation of granulation tissue [31].

Collagen is the predominant extracellular protein in the granulation tissue of a healing wound and there is a rapid increase in the synthesis of this protein in the wound area soon after an injury. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of this hydroxyproline, therefore, has been used as an index of collagen turnover. The biochemical data of dead space wound study showed an increase in wet tissue weight and protein per g tissue in BME treated groups. The increased hydroxyproline content in the dead space wounds has indicated faster collagen turnover leading to rapid healing with concurrent increase in the breaking strength of the treated wounds. Hexosamine and hexuronic acids are matrix molecules, which act as ground substratum for the synthesis of new extracellular matrix. The glycosaminoglycans are known to stabilize the collagen fibres by enhancing electrostatic and ionic interactions with it and possibly control their ultimate alignment and characteristic size. Their ability to bind and alter protein-protein interactions has identified them as important determinants of cellular responsiveness in development, homeostasis, and disease [32]. In our study, hexuronic acid and hexosamine concentrations which are the components of glycosaminoglycans were significantly increased with BME when compared with control indicating stabilization of collagen fibres.

Table 3: Effect of BME and VTE on dry granulation tissue, protein, hydroxyproline, hexosamine, and hexuronic acid content.

Oral treatment (mg/kg, od × 10 day)	Dry tissue mg/100 g bw	Protein mg/g dry tissue	Connective tissue parameters		
			Hydroxyproline μg/mg protein	Hexosamine μg/mg protein	Hexuronic Acid μg/mg protein
Control 0.5% CMC	71.2 ± 3.80	244.1 ± 14.4	145.7 ± 12.6	86.6 ± 7.02	20.8 ± 3.94
BME 25	85.0 ± 3.70[a]	288.4 ± 10.3[a]	204.2 ± 9.44[b]	122.1 ± 7.70[b]	52.6 ± 2.90[c]
VTE 200	92.2 ± 2.0[c]	295.5 ± 17.0[a]	191.1 ± 11.2[b]	126.2 ± 8.97[b]	46.0 ± 2.86[c]

values are mean ± SEM of 6 rats in each group. [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$ compared to respective control group (statistical analysis was done by one-way analysis of variance followed by Dunnett's test for multiple comparisons).

Experimental and clinical lines of evidence suggest that chronic wound undergoes substantial oxidative stress by neutrophils-derived oxidants and MPO activity, both of which contribute markedly to tissue damage during chronic wound inflammation [33]. Over production of reactive oxygen species (ROS) results in oxidative stress thereby causing cytotoxicity and delayed wound healing and elimination of ROS could be an important strategy in healing of chronic wounds [4]. Therefore, estimation of antioxidants like GSH, SOD, and CAT in granulation tissues is relevant because the antioxidants have been reported to hasten wound healing by decreasing the free radicals [34]. Our studies on the antioxidants, free radicals, and MPO status revealed that BME had significant antioxidant activity, reduced MPO and free radicals stress, helped to prevent inflammation and oxidative damage, and promoted the healing process.

The preliminary phytochemical analysis of *Bacopa monniera* (BM) revealed the presence of saponins, glycosides, and alkaloids [35]. Animal research has shown that the phytoconstituents present in BM extract modulate the expression of certain enzymes involved in generation and scavenging of reactive oxygen species in the brain [7]. BM's antioxidant properties and its ability to balance superoxide dismutase and catalase levels were postulated to account for this effect [8]. BM by modulating the extent of lipid peroxidation and enhancing the antioxidant status has DNA protective effects; hence, it is believed to prevent cell damage [36]. Extracts of BM have been reported to have a broad spectrum of antibacterial activity [37], which seemed to have beneficial effects on wound healing. BME was found to show antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis*,and *Pseudomonas aeruginosa* and least MIC was found with BME against *Staphylococcus aureus*.

5. Conclusion

Thus, in our present study involving three different wound models, which included observation of different physical, histological, biochemical parameters, and antimicrobial activity, indicated the wound healing activity in the 50% ethanolic extract of dried whole plant of *Bacopa monniera*. The healing effects seemed to be due to decreased free radical generated tissue damage, promoting effects on antioxidant status, faster collagen deposition, and other connective tissue constituent formation, and antibacterial activity.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

S. Murthy and M. K. Gautam equally contributed to the paper.

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Research Article

Effects of an *Agaricus blazei* Aqueous Extract Pretreatment on Paracetamol-Induced Brain and Liver Injury in Rats

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The action of an *Agaricus blazei* aqueous extract pretreatment on paracetamol injury in rats was examined not only in terms of the classical indicators (e.g., levels of hepatic enzymes in the plasma) but also in terms of functional and metabolic parameters (e.g., gluconeogenesis). Considering solely the classical indicators for tissue damage, the results can be regarded as an indication that the *A. blazei* extract is able to provide a reasonable degree of protection against the paracetamol injury in both the hepatic and brain tissues. The *A. blazei* pretreatment largely prevented the increased levels of hepatic enzymes in the plasma (ASP, ALT, LDH, and ALP) and practically normalized the TBARS levels in both liver and brain tissues. With respect to the functional and metabolic parameters of the liver, however, the extract provided little or no protection. This includes morphological signs of inflammation and the especially important functional parameter gluconeogenesis, which was impaired by paracetamol. Considering these results and the long list of extracts and substances that are said to have hepatoprotective effects, it would be useful to incorporate evaluations of functional parameters into the experimental protocols of studies aiming to attribute or refute effective hepatoprotective actions to natural products.

1. Introduction

The mushrooms have generally been considered functional foods and, consequently, important sources of bioactive compounds, with the β-glucans, terpenes, phenolics, steroids, and nucleosides being so far the most important. These compounds are said to exert several effects, the most prominent being the antitumoral, antimutagenic, anticytotoxic, antimicrobial, antioxidant, hepatoprotective, anti-inflammatory, and hypoglycemic and antidislipidemic actions [1, 2]. *Agaricus blazei* Murrill is a basidiomycete whose popular name is "sun mushroom" and which has become the subject of great interest, due to its nutritional value and pharmacological properties [3]. In Brazil *A. blazei* is greatly consumed in the form of concentrated extracts or teas and popularly used against a variety of diseases such as diabetes, atherosclerosis, hypercholesterolemia, and heart disease [3–7].

Liver injury is a frequent and multivariate phenomenon which can have dangerous and even fatal consequences. Liver damage involves in most cases oxidative stress and is characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [8]. For this reason, the search for hepatoprotectors has been intense during the last decades. Hepatoprotection has been attributed to many substances or natural extracts. This includes several aqueous extracts of mushrooms, as for example, *Ganoderma lucidum* [9] and *Ganoderma tsugae* [10]. *A. blazei* extracts (1500 mg/day) have been reported to normalize the liver functions (aminotransferases) of patients with hepatitis B when given at doses of 1500 mg/day [11]. It has also been reported that the mushroom is able to diminish the hepatotoxic effects induced by diethylnitrosamine in rats [12]. More recently it has been shown that a hydroalcoholic extract of *A. blazei* exerts several purinergic effects in the

rat liver [13]. These effects were attributed to the various nucleotides and nucleosides which have been demonstrated to exist in *A. blazei* extracts.

Taken as a whole the investigations that have so far been done with *A. blazei* do not allow much more than preliminary conclusions about the possibilities of the mushroom as a general protective or therapeutic agent [6]. For this reason we decided to start a systematic investigation of the possible protective action of *A. blazei.* The experimental model that was chosen was the tissue injury induced in rats by paracetamol [14]. The latter, a commonly used analgesic, effectively reduces fever and mild to moderate pain and is considered to be safe at therapeutic doses. Paracetamol overdose, however, causes severe hepatotoxicity that leads to liver failure in both humans and experimental animals [15–17]. In all previous studies in which the influence of edible mushroom extracts on paracetamol injury was investigated, the measurements were concentrated on the liver in which histology, enzyme markers and oxidative stress markers were assayed [14]. In the present study attempts were made to extend these measurements to other parameters and other tissues in addition to the liver. For this purpose the actions of paracetamol and an *A. blazei* extract on several parameters in the brain tissue were also investigated. This tissue was chosen taking into account the high content in nucleosides of the *A. blazei* extract [13] and because there are reports about beneficial effects of these compounds on the neural tissue [18]. Moreover, in the liver, the investigations were expanded in order to encompass also functional parameters such as gluconeogenesis, oxygen consumption, and other parameters related to lipid metabolism. The results are hoped to contribute for a better understanding of the action of mushroom extracts on the liver and brain.

2. Materials and Methods

2.1. Materials. Commercial diagnostic kits were used for the plasma analyses. All other chemicals used were in the purest form available commercially.

2.2. Preparation of the A. blazei Extract. Fruiting bodies (basidiocarps) of *A. blazei* Murrill were obtained from a local producer in Maringá, PR, Brazil, in Spring 2009. The dried basidiocarps were milled until obtaining a fine powder. The samples (10 g) were extracted by stirring with 100 mL of water (28°C) at 130 rpm for 3 hours and filtered through Whatman paper n°1. The extraction was repeated three times. The filtrates (yield 50%) were lyophilized and stored in freezer until use.

2.3. Animals, Experimental Protocol, and Tissue Preparation. Male albino rats (*Wistar*), weighing 250–300 g, were fed *ad libitum* with a standard laboratory diet (Purina). The rats were maintained in automatically timed light and dark cycles of 12 hours. The present study was approved by the Ethics Committee for the Use of Experimental Animals of the Universidade Estadual de Maringá.

Rats were distributed randomly into four groups. To the rats of group I (the control group) saline (0.9% NaCl) was administered orally each day during 21 days. Rats of group II were treated orally with the *A. blazei* extract during 21 days; the dosage was 200 mg × (kg body weight)$^{-1}$× day^{-1}. Group III was the hepatotoxicity group to which paracetamol (Tylenol) was given orally as a single dose of 2 g × (kg body weight)$^{-1}$. Group IV was treated with the *A. blazei* extract in the same way as group II during 21 days; at this time paracetamol was given as a single dose of 2 g × (kg body weight)$^{-1}$. Animals were sacrificed 48 h after the single dose of paracetamol or at the corresponding times for those that did not receive paracetamol by intraperitoneal injection of sodium thiopental (50 mg/body weight). Blood samples were collected and plasma was separated by centrifugation at 2500 rpm for 15 minutes for the hepatic marker enzyme assays. Livers, and brains were excised, weighed, frozen in liquid nitrogen and stored until use.

For the analyses the frozen livers and brains were weighed and homogenized in 10 volumes of 0.1 M potassium phosphate buffer (pH 7.4). This homogenate was used for determining the oxidative stress parameters lipid peroxidation (TBARS), reduced glutathione (GSH), and protein reduced thiol groups. For the determination of enzymatic activities (CAT, SOD, GR, and GPx) and reactive oxygen species (ROS), the homogenate was centrifuged at 10000 g for 15 min and the resulting supernatants were used for the assays. Protein concentration was measured with the Folin-Ciocalteu reagent, using bovine serum albumin as a standard [19].

2.4. Plasma Hepatic Markers. Plasma samples were assayed for the hepatic marker enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). The results were expressed in international units (IU) per liter. Total and conjugated bilirubin and albumin were also assayed. In all cases commercial diagnostic kits were used.

2.5. Plasma Paracetamol. Plasma paracetamol was evaluated in rats from groups III and IV. The rats were anesthetized with sodium thiopental (50 mg/kg) and blood was collected from the cava vein (5 mL). After collection blood was centrifuged for 4000 rpm during 10 minutes at 4°C. The supernatant was used for paracetamol extraction [20, 21]. The latter was done by adding 6 volumes of ethylacetate. This addition was followed by vigorous shaking during two minutes. An aliquot of 1 mL from the organic phase was used for the spectrophotometric paracetamol determination at 290 nm (ε = 0.54 mM^{-1} cm^{-1}) [20]. A plasma extract from rats of group I was used as the blank.

2.6. Oxidative Stress Parameters. Lipid peroxidation was evaluated by means of the TBARS assay (thiobarbituric acid reactive substances) [22, 23]. The amount of lipoperoxides was calculated from the standard curve prepared with 1,1′,3,3′tetraethoxy-propane and values were expressed as nmol (mg protein)$^{-1}$.

A fluorometric assay was used to determine the relative levels of different reactive oxygen species (ROS) [24]. The formation of the oxidized fluorescent derivative 2′,7′-dichlorofluorescein (DCF) was measured with a fluorescence spectrophotometer using excitation and emission wavelengths at 504 and 529 nm, respectively. The results were expressed as nmol of DCF formed per mg of protein (nmol/mg protein).

Reduced glutathione (GSH) levels were measured spectrofluorimetrically (excitation at 350 nm and emission at 420 nm) by means of the o-phthalaldehyde assay as described previously [25]. The results were expressed as nmol of GSH per mg of protein (nmol/mg protein).

The levels of protein thiol groups were determined using 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) [26, 27]. The reaction product was measured spectrophotometrically at 412 nm and the molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ was used to express the results as nmol (mg protein)$^{-1}$.

2.7. Antioxidant Enzymes. The catalase (CAT) activity was estimated by measuring the change in absorbance at 240 nm using H_2O_2 as substrate and expressed as μmol min^{-1} (mg protein)$^{-1}$ [28]. The glutathione reductase (GR) activity was estimated by measuring the change in absorbance at 340 nm using NADPH and GSSG as substrates and expressed as nmol min^{-1} (mg protein)$^{-1}$ [29]. The superoxide dismutase (SOD) activity was estimated by its capacity of inhibiting the pyrogallol autooxidation in alkaline medium. The latter was measured at 420 nm [30]. One SOD unit (U) was considered the quantity of enzyme that was able to promote 50% inhibition and the results were expressed as U (mg protein)$^{-1}$. The glutathione peroxidase (GPx) activity was estimated by measuring the change in absorbance at 340 nm due to NADPH consumption in the presence of H_2O_2, GSH, and glutathione reductase and expressed as nmol min^{-1} (mg protein)$^{-1}$ [31, 32].

2.8. Liver Perfusion. Hemoglobin-free, non-recirculating perfusion was done according to the technique described elsewhere [33, 34]. For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The constant flow was provided by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted between 30 and 34 mL/min, depending on the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine-serum albumin, saturated with a mixture of oxygen and carbon dioxide (95 : 5) by means of a membrane oxygenator with simultaneous temperature adjustment (37°C). In the effluent perfusion fluid the following compounds were assayed by means of standard enzymatic procedures: glucose, lactate, pyruvate, ammonia, and urea [29]. The oxygen concentration in the outflowing perfusate was monitored continuously, employing a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate [34]. Metabolic rates were calculated from input-output differences and the total flow rates and were referred to the wet weight of the liver.

2.9. Hepatic Glycogen and Lipids. The hepatic glycogen was determined enzymatically after freeze-clamping freshly isolated livers from anesthetized fed rats (sodium thiopental, 50 mg/kg) [29]. Portions of approximately 2 g of the freeze-clamped tissue were extracted with 10 mL of 0.6 M perchloric acid. Glycogen in the extract was hydrolyzed using amyloglucosidase. The reaction mixture contained 0.1 M potassium hydrogen carbonate, 0.135 M acetate buffer (pH 4.8), 0.75 units of amyloglucosidase, and 200 μL of the extract. After 2 hours incubation at 40°C the reaction was stopped by adding 500 μL of 0.6 M perchloric acid. The mixture was centrifuged and neutralized with potassium hydrogen carbonate and free glucose was determined with a commercial kit. The results were expressed as μmol glucosyl units per gram liver.

Total lipids were extracted from freshly isolated livers and determined gravimetrically [35]. Portions of approximately 5 g tissue were freeze-clamped with liquid nitrogen and subjected to the successive extraction procedures which culminated with a chloroformic lipidic phase [35]. This phase was transferred to a previously weighed beaker and placed in a stove at 45°C for approximately 3 hours. After chloroform evaporation the beaker was weighed and the results expressed as percentage of total lipids per gram liver. For the determination of triacylglycerols, total cholesterol and high-density lipoprotein cholesterol (HDL), the total lipids were dissolved in 1.0 mL chloroform plus 2.0 mL isopropanol, freeze-dried, and stored in freezer. Determinations of triacylglycerols, total cholesterol, and HDL were made using commercial colorimetric-enzymatic kits. The results were expressed as μg per mg total lipids.

2.10. Liver Histology. Samples of the left liver lobe ($n = 5$) were fixed in Bouin for 24 h and transferred to 70% ethanol. After dehydration with solutions of successively higher ethanol proportions (80%, 90%, and 100%) and additional 12 hours in xylol the samples were embedded in parafin. These preparations were cut into 7 μm thick semiserial sections and submitted to hematoxylin-eosin staining. The sections were analyzed under microscope. Histological damage was scored into four damage levels [36]: absent (0), mild (+), moderate (++), and severe (+++). Besides this qualitative analysis, the liver degeneration was evaluated using a high resolution digital camera coupled to a microscope. An appropriate software was used to estimate the percentage of liver degeneration.

2.11. Statistical Analysis. All analyses were performed in triplicate. The data were expressed as means ± standard errors of the man (SEM). One-way analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls testing was done; $P < 0.05$ was adopted as the criterion of significance.

3. Results

3.1. Plasma Paracetamol Levels. The plasma paracetamol levels were measured in rats of groups III and IV during the first 4 hours after administration. The results are shown in Figure 1. In rats of group III (only paracetamol administration) the plasma paracetamol levels were as high as 6.82 mM at one

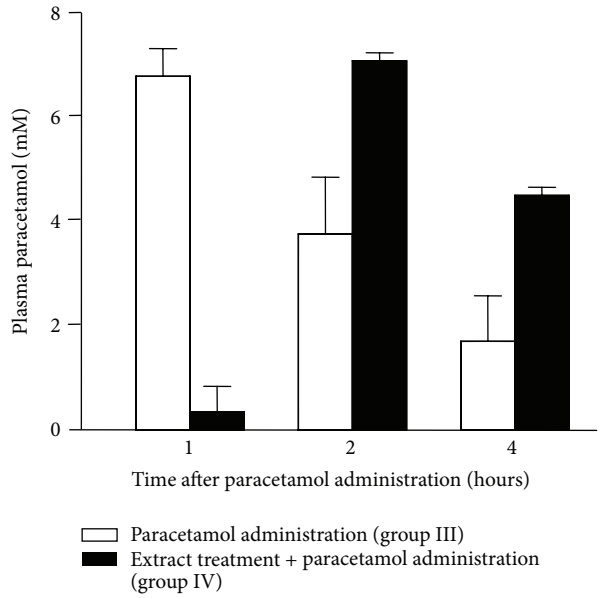


Figure 1: Plasma paracetamol levels at various times after oral administration of 2 g × (kg body weight)$^{-1}$. The four experimental groups (III and IV) are identified on the top. Experimental details can be found in Section 2. Data are means plus mean standard errors of three independent determinations.

hour after administration, but they declined progressively during the next hours. In the rats of group IV (*A. blazei* pretreatment and paracetamol injury), the highest paracetamol level (7.11 mM) was found two hours after administration. A declining tendency of the plasma levels of paracetamol was also apparent for group IV, even though the decline was clearly delayed with respect to group III.

3.2. Histopathological Evaluations. Figure 2 shows representative photomicrographs of liver sections stained with hematoxylin and eosin. Table 1 presents the results of the morphological analyses in addition to the liver weights. Groups I and II present the standard histological organization. The sections presented well defined capsules and septa of connective tissue, portal tracts, and central veins with the polyhedral shaped hepatic cells arranged in the form of cords between the sinusoids. The other two groups, III and IV, showed a damaged liver architecture (twisted cords of hepatocytes and veins, disorganized parenchyma), but especially inflammatory infiltrates around the central veins, with the presence of neutrophils and monocytes. In some specimens inflammatory foci around the hepatic paren-chyma were visible. All these observations reflect in the liver damage scores listed in Table 1 and in the estimate of liver degeneration. Both parameters were not significantly modified by the *A. blazei* pretreatment. The fresh liver weight presented a tendency to increase in consequence of the paracetamol injury, but statistical significance at the 5% level was lacking.

3.3. Plasma Hepatic Markers. The levels of enzyme markers that are generally considered as indicative of liver damage are listed in Table 2. The *A. blazei* extract treatment did not produce significant alterations in the parameters listed in Table 2. The paracetamol injury, however, increased all the enzymes that were measured: AST, ALT, LDH, and ALP. It also increased the conjugated bilirubin levels with a nonsignificant tendency for increasing also total bilirubin. The albumin concentration was not affected by the paracetamol injury. The *A. blazei* extract pretreatment reduced considerably the release of hepatic enzymes induced by paracetamol. The lactate dehydrogenase levels, particularly, were close to normal in group IV. The *A. blazei* pretreatment also reduced the appearance of conjugated bilirubin which had been greatly increased by the paracetamol injury.

3.4. Oxidative Stress Assays. Table 3 lists the results of several oxidative stress markers in both brain and liver. Paracetamol administration had a marked influence on the levels of thiobarbituric acid reactive substances (TBARS). In both tissues the TBARS were doubled or nearly so upon paracetamol injury. Remarkably, also in both tissues, the *A. blazei* extract pretreatment effectively prevented the extra TBARS formation induced by paracetamol. The GSH levels suffered a small reduction upon paracetamol injury, an effect that was also prevented by the *A. blazei* extract pretreatment. For the other parameters that were measured, ROS and protein thiol groups, statistical significance was lacking for the eventually small modifications caused by paracetamol or *A. blazei* pretreatment. It must equally be mentioned that the *A. blazei* pretreatment alone was without effect on the variables listed in Table 3.

3.5. Antioxidant Enzyme Assays. *A. blazei* pretreatment did not affect the activites of the four enzymes that were measured in the present work in both brain and liver, as shown in Table 4. Paracetamol injury, however, diminished significantly the activities of catalase and superoxide dismutase in both liver and brain; glutathione reductase and glutathione peroxidase were diminished only in the brain, though to a relatively small extent. The diminution of the catalase activity by paracetamol was quite pronounced: 55% in the liver and 25% in the brain. It is noteworthy that the catalase activity in the brain performs only a small fraction of the activity in the liver (1.5%). In *A. blazei* pretreated rats paracetamol was considerably less effective in promoting the reduction of the enzymatic activities. This is especially true for catalase, whose activities were close to normal in paracetamol injured + *A. blazei* pretreated rats.

3.6. Liver Metabolism. Liver metabolism was measured in order to verify if paracetamol injury also affects metabolic routes in the liver and if *A. blazei* pretreatment has some protective action. Alanine metabolism was chosen because the use of this substrate allows measuring simultaneously a biosynthetic route (gluconeogenesis) and evaluating both carbon and nitrogen metabolism. Food was withdrawn from the rats 18 h before the experiments in order to minimize the interference of glycogen catabolism on gluconeogenesis [37]. Figure 3 shows the time courses of glucose and lactate

Table 1: Weight and morphological characteristics of rat livers from the four experimental groups used in the present study. Means and observations were derived from 5 animals in each group. Histological damage was scored into four damage levels: absent (0), mild (+), moderate (++), and severe (+++). The software Image-Pro Plus 4.5 was used to estimate the percentage of liver degeneration. For additional experimental details and a more complete description of the experimental groups see Section 2.

Groups	Liver weights	Morphological aspects	Liver damage scores				Estimates of liver degeneration
			0	+	++	+++	
I, control	9.02 ± 0.69	Uniformly red and soft consistency	5	0	0	0	—
II, *A. blazei* pretreatment	9.16 ± 1.17	Uniformly red and soft consistency	5	0	0	0	—
III, paracetamol injury	11.50 ± 0.86	Larger, whitish, and with nodules	0	1	3	1	11.78%
IV, *A. blazei* pretreatment + paracetamol injury	10.40 ± 1.61	Larger, whitish, and with nodules	1	0	3	1	9.89%

Table 2: Effects of paracetamol injury and *A. blazei* pretreatment on plasma enzyme activities, albumin levels, and bilirubin concentration. For experimental details see Section 2.

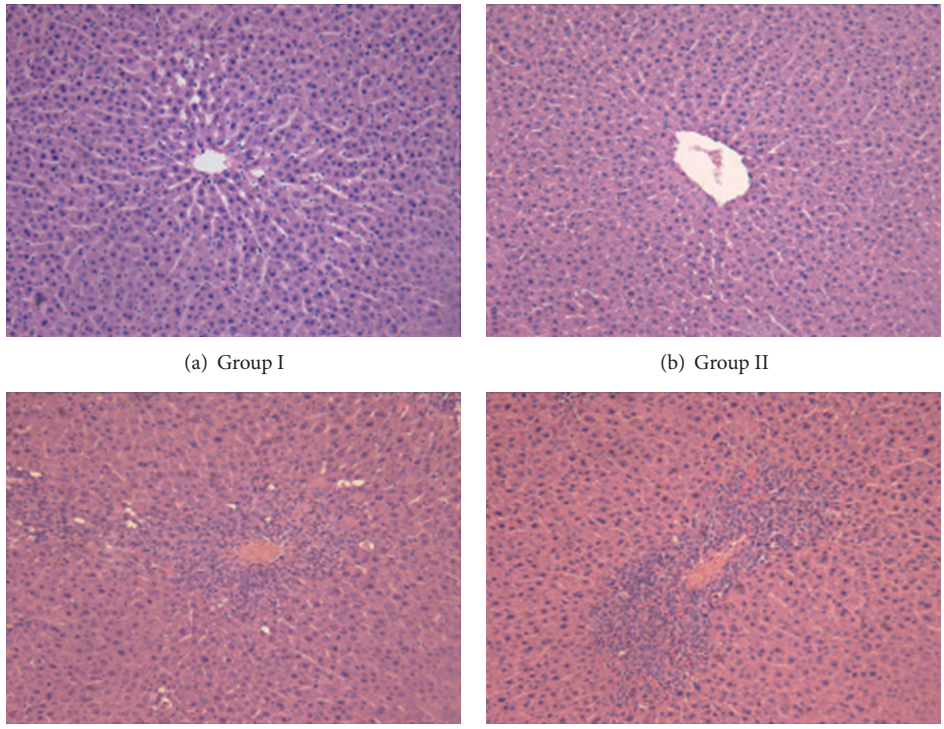
Parameters	Groups			
	I, control	II, *A. blazei* pretreatment	III, paracetamol injury	IV, *A. blazei* pretreatment + paracetamol injury
Aspartate aminotransferase (AST, U/L)	74.6 ± 9.3 ($n = 4$)	71.6 ± 4.0 ($n = 4$)	512.9 ± 62.7 ($n = 4$)[a,b,c]	246.6 ± 65.2 ($n = 4$)[d,e]
Alanine aminotransferase (ALT, U/L)	65.3 ± 4.2 ($n = 5$)	63.7 ± 2.6 ($n = 4$)	447.0 ± 52.0 ($n = 4$)[a,b,c]	280.2 ± 64.0 ($n = 4$)[d,e]
Lactate dehydrogenase (LDH, U/L)	248.9 ± 12.5 ($n = 4$)	227.7 ± 38.1 ($n = 4$)	531.0 ± 36.8 ($n = 5$)[a,b,c]	304.4 ± 24.7 ($n = 4$)
Alkaline phosphatase (ALP, U/L)	74.6 ± 14.8 ($n = 4$)	72.6 ± 3.6 ($n = 4$)	174.1 ± 16.2 ($n = 5$)[a,b,c]	123.3 ± 5.6 ($n = 5$)[d,e]
Albumin (g/L)	2.37 ± 0.09 ($n = 5$)	2.21 ± 0.14 ($n = 5$)	2.48 ± 0.20 ($n = 4$)	2.22 ± 0.10 ($n = 6$)
Total bilirubin (mg/dL)	0.53 ± 0.01 ($n = 4$)	0.35 ± 0.01 ($n = 4$)	0.80 ± 0.01 ($n = 5$)[b]	0.64 ± 0.11 ($n = 6$)[e]
Conjugated bilirubin (mg/dL)	0.07 ± 0.01 ($n = 4$)	0.07 ± 0.01 ($n = 4$)	0.24 ± 0.01 ($n = 4$)[a,b,c]	0.17 ± 0.01 ($n = 6$)[d,e]

Significant differences ($P \leq 0.05$), according to one-way ANOVA followed by Student-Newman-Keuls post-hoc testing, are identified by the superscript letters as follows: [a]III versus I; [b]III versus II; [c]III versus IV; [d]IV versus I; [e]IV versus II.

Table 3: Effects of paracetamol injury and *A. blazei* pretreatment on oxidative stress indicators in the liver and brain tissues. For experimental details see Section 2.

Parameters	Organ	I, control	II, *A. blazei* pretreatment	III, paracetamol injury	IV, *A. blazei* pretreatment + paracetamol injury
TBARS (nmol mg^{-1})	Liver	1.05 ± 0.19 ($n = 4$)	1.01 ± 0.18 ($n = 4$)	2.08 ± 0.23 ($n = 3$)[a,b,c]	1.08 ± 0.16 ($n = 4$)
	Brain	2.64 ± 0.08 ($n = 6$)	2.73 ± 0.11 ($n = 6$)	3.29 ± 0.21 ($n = 6$)[a,b,c]	2.61 ± 0.18 ($n = 6$)
ROS (nmol mg^{-1})	Liver	1.96 ± 0.38 ($n = 3$)	2.31 ± 0.28 ($n = 3$)	2.48 ± 0.48 ($n = 3$)	2.23 ± 0.47 ($n = 4$)
	Brain	8.31 ± 0.91 ($n = 5$)	8.63 ± 0.66 ($n = 5$)	9.62 ± 0.69 ($n = 4$)	8.61 ± 0.74 ($n = 4$)
GSH (nmol mg^{-1})	Liver	13.66 ± 0.65 ($n = 4$)	13.14 ± 0.71 ($n = 4$)	9.76 ± 0.91 ($n = 4$)[f,g]	12.66 ± 2.02 ($n = 3$)
	Brain	8.82 ± 0.19 ($n = 6$)	8.79 ± 0.61 ($n = 6$)	7.31 ± 0.23 ($n = 5$)[h]	8.28 ± 0.45 ($n = 8$)
Protein thiol groups (nmol mg^{-1})	Liver	115.3 ± 14.0 ($n = 4$)	122.6 ± 14.7 ($n = 4$)	80.7 ± 7.4 ($n = 4$)	101.1 ± 2.0 (n =4)
	Brain	95.2 ± 5.5 ($n = 5$)	95.0 ± 4.8 ($n = 5$)	84.4 ± 3.6 ($n = 7$)	84.4 ± 3.5 ($n = 6$)

Significant differences ($P \leq 0.05$), according to one-way ANOVA followed by Student-Newman-Keuls post-hoc testing, are identified by the superscript letters as follows: [a]III versus I; [b]III versus II; [c]III versus IV. Significant differences according to Student's t-test are given by the codes: [f]$P = 0.013$ for III versus I; [g]$P = 0.026$ for III versus I; [h]$P < 0.001$ for III versus I.



(a) Group I (b) Group II

(c) Group III (d) Group IV

Figure 2: Photomicrographs of rat liver sections stained with hematoxylin and eosin showing the central vein (200x). The four experimental groups (I, II, III, and IV) are identified. Legends for the experimental groups: I, control; II, *A. blazei* pretreatment; III, paracetamol injury; IV, *A. blazei* pretreatment + paracetamol injury.

Table 4: Effects of paracetamol injury and *A. blazei* pretreatment on antioxidant enzyme activities in the liver and brain tissues. For experimental details see Section 2.

Enzymes	Organ	I, control	II, *A. blazei* pretreatment	III, paracetamol injury	IV, *A. blazei* pretreatment + paracetamol injury
Catalase (μmol min^{-1} mg^{-1})	Liver	1078.8 ± 75.1 ($n = 4$)	942.5 ± 104.1 ($n = 3$)	597.0 ± 68.2 ($n = 4$)^{a,b,c}	898.3 ± 33.9 ($n = 4$)
	Brain	15.97 ± 0.79 ($n = 5$)	16.79 ± 1.21 ($n = 5$)	12.00 ± 0.98 ($n = 6$)^{a,b}	14.56 ± 0.77 ($n = 5$)
Superoxide dismutase (units mg^{-1})	Liver	4.29 ± 0.14 ($n = 4$)	4.3 ± 0.42 ($n = 4$)	2.97 ± 0.17 ($n = 4$)^{a,b}	3.30 ± 0.22 ($n = 4$)^{d,e}
	Brain	2.03 ± 0.13 ($n = 5$)	1.97 ± 0.09 ($n = 5$)	1.46 ± 0.09 ($n = 6$)^{a,b,c}	1.78 ± 0.10 ($n = 7$)
Glutathione reductase (nmol min^{-1} mg^{-1})	Liver	36.11 ± 3.11 ($n = 4$)	38.38 ± 2.04 ($n = 4$)	46.25 ± 5.85 ($n = 3$)	47.25 ± 1.82 ($n = 3$)
	Brain	14.34 ± 0.73 ($n = 6$)	15.58 ± 0.99 ($n = 6$)	12.76 ± 0.43 ($n = 8$)^{b,c}	14.99 ± 0.44 ($n = 8$)
Glutathione peroxidase (nmol min^{-1} mg^{-1})	Liver	107.2 ± 5.7 ($n = 3$)	133.8 ± 9.0 ($n = 3$)	122.3 ± 16.2 ($n = 3$)	139.7 ± 16.1 ($n = 3$)
	Brain	33.0 ± 1.3 ($n = 5$)	31.5 ± 2.1 ($n = 5$)	27.4 ± 0.5 ($n = 6$)^{a}	30.8 ± 0.7 ($n = 7$)

Significant differences ($P \leq 0.05$), according to one-way ANOVA followed by Student-Newman-Keuls post-hoc testing, are identified by the superscript letters as follows: ^{a}III versus I; ^{b}III versus II; ^{c}III versus IV; ^{d}IV versus I; ^{e}IV versus II.

production in response to alanine infusion in perfused rat livers belonging to the four experimental groups used in the present work. The variables shown in Figure 3 were those more strongly affected by both paracetamol and *A. blazei*. Figure 3 also illustrates the experimental protocol that was employed. Glucose release, lactate production, and several additional parameters were measured before (basal rates) and during alanine infusion. Livers from fasted rats when perfused with a substrate-free medium respire mainly at the expense of endogenous fatty acids [33]. Glycogen

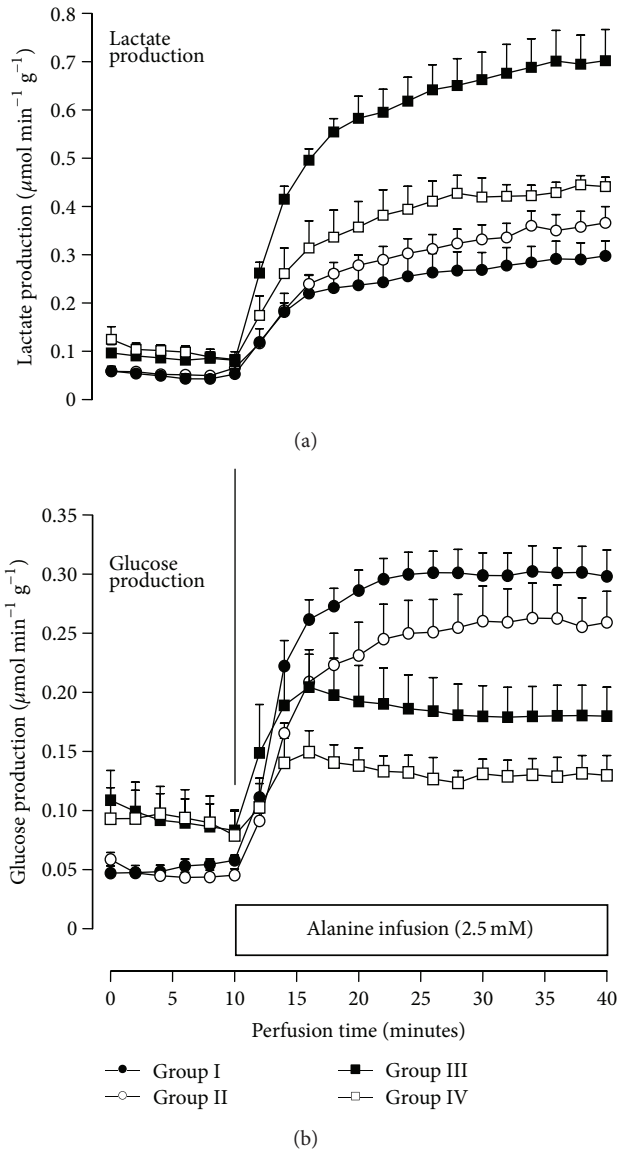


FIGURE 3: Time course of lactate (a) and glucose (b) production from alanine in the perfused liver from rats of four experimental groups. Livers from fasted rats were perfused as described in Section 2. Alanine was infused during 30 minutes (10 to 40 minutes perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Group I, control; group II, *A. blazei* pretreatment; group III, paracetamol injury; group IV, *A. blazei* pretreatment + paracetamol injury.

content of these livers is quite small, a situation that also reflects in proportionally small rates of metabolite release [37, 38]. Even so it is noteworthy to mention that these residual rates of glucose release and lactate production were more pronounced in livers from paracetamol injured rats, namely, groups III and IV. The basal rates of glucose release in groups III and IV were 0.087 ± 0.011 and $0.094 \pm 0.014\ \mu\text{mol min}^{-1}\ \text{g}^{-1}$, respectively. These rates differ from those in groups I and II ($P < 0.05$) which were equal to 0.048 ± 0.005 and $0.047 \pm 0.005\ \mu\text{mol min}^{-1}\ \text{g}^{-1}$, respectively. The same was found for lactate production: 0.086 ± 0.011 and $0.10 \pm 0.02\ \mu\text{mol min}^{-1}\ \text{g}^{-1}$, respectively, for groups III and IV, but 0.050 ± 0.002 and $0.046 \pm 0.005\ \mu\text{mol min}^{-1}\ \text{g}^{-1}$, respectively, for groups I and II ($P < 0.05$). These observations mean a more accentuated glycogen degradation in livers from paracetamol injured rats, a phenomenon that was not affected by the *A. blazei* pretreatment. Upon alanine infusion (Figure 3) both lactate and glucose production increased due to alanine transformation. The excess glucose production is now the consequence of gluconeogenesis and it is quite apparent that the paracetamol injury had a strong negative effect (Figure 3(b)). *A. blazei* pretreatment alone also tended to be inhibitory, an effect that was further enhanced in rats with paracetamol injury. Lactate production derived from alanine transformation, on the other hand, was greatly increased by the paracetamol injury (Figure 3(a)). The *A. blazei* pretreatment was able to prevent this action in part.

Table 5 complements the analysis of the perfusion experiments in two ways: (a) the metabolic rates are given in terms of the increments caused by alanine; that is, they represent only alanine metabolism; (b) all other variables that were measured are represented in addition to glucose and lactate production. The basal rates of the variables other than glucose and lactate production were not different in the four experimental groups (not shown). They were generally very small with the exception of oxygen uptake which was always around $2\ \mu\text{mol min}^{-1}\ \text{g}^{-1}$. Table 5 reveals that, in addition to lactate production, the paracetamol injury also increased pyruvate and ammonia productions from alanine. Urea production was not affected by paracetamol, but it was diminished by the *A. blazei* pretreatment. The oxygen uptake increment caused by alanine was not significantly affected by paracetamol, but there was a tendency toward diminution in livers from *A. blazei* pretreated rats. With the exception of the alanine derived lactate production the *A. blazei* pretreatment did not prevent the metabolic alterations caused by paracetamol.

3.7. Hepatic Levels of Glycogen and Lipids. The hepatic glycogen and lipid contents are listed in Table 6. Livers from fed rats were utilized. As expected from previous studies [39], the paracetamol injury diminished the glycogen levels (group III). *A. blazei* pretreatment alone was without effect on the glycogen levels, and it also failed to prevent the depleting action of paracetamol in a significant way (group IV).

The paracetamol injury did not affect the total fat content of the liver nor did *A. blazei* pretreatment have any action on this variable. The same can be said about the hepatic triglyceride contents. The hepatic cholesterol content, however, which comprises only a small fraction of the total lipid content, was considerably increased in rats injured by paracetamol (+213%). This action was effectively prevented by the *A. blazei* pretreatment. The HDL-cholesterol levels were also increased by paracetamol (+174%). Singularly, the *A. blazei* pretreatment also prevented this increase in spite of the fact that the *A. blazei* pretreatment alone also caused a substantial increase in the HDL-cholesterol levels.

Table 5: Effects of paracetamol injury and *A. blazei* pretreatment on metabolic parameters of perfused rat livers metabolizing exogenously supplied alanine. Livers from fasted rats were perfused according to the experimental protocol illustrated by Figure 2. For additional experimental details and see Section 2.

Metabolic fluxes (μmol min^{-1} g^{-1})	I, control	II, *A. blazei* pretreatment	III, paracetamol injury	IV, *A. blazei* pretreatment + paracetamol injury
Lactate production	0.31 ± 0.03 (n = 5)	0.25 ± 0.03 (n = 4)	0.62 ± 0.05 (n = 5)^{a,b,c}	0.35 ± 0.02 (n = 3)
Pyruvate production	0.13 ± 0.02 (n = 5)	0.082 ± 0.030 (n = 4)	0.29 ± 0.05 (n = 5)^{a,b}	0.20 ± 0.02 (n = 3)
Glucose production	0.25 ± 0.02 (n = 5)	0.21 ± 0.02 (n = 4)	0.092 ± 0.020 (n = 5)^{a,b}	0.060 ± 0.030 (n = 3)^{d,e}
Oxygen consumption	0.39 ± 0.06 (n = 5)	0.23 ± 0.05 (n = 4)	0.32 ± 0.07 (n = 5)	0.29 ± 0.03 (n = 3)
Ammonia production	0.058 ± 0.010 (n = 5)	0.085 ± 0.030 (n = 4)	0.20 ± 0.03 (n = 5)^{a,b}	0.16 ± 0.01 (n = 3)^{d}
Urea production	0.28 ± 0.01 (n = 5)	0.12 ± 0.01 (n = 4)^{f,g}	0.23 ± 0.03 (n = 5)^{b}	0.25 ± 0.05 (n = 3)

Significant differences ($P \leq 0.05$), according to one-way ANOVA followed by Student-Newman-Keuls post-hoc testing, are identified by the superscript letters as follows: ^{a}III versus I; ^{b}III versus II; ^{c}III versus IV; ^{d}IV versus I; ^{e}IV versus II; ^{f}II versus I; ^{g}II versus IV.

Table 6: Effects of paracetamol injury and *A. blazei* pretreatment on hepatic glycogen and lipid levels. Livers from fed rats were utilized. For additional experimental details see Section 2.

Parameters	I, control	II, extract treatment	III, paracetamol injury	IV, *A. blazei* pretreatment + paracetamol injury
Hepatic glycogen (μmol glucosyl units/g liver)	260.4 ± 16.9 (n = 5)	273.3 ± 7.4 (n = 6)	202.3 ± 4.5 (n = 6)^{a,b}	223.8 ± 21.5 (n = 5)^{d}
Total lipids (g/100 g liver)	3.66 ± 0.13 (n = 8)	3.62 ± 0.16 (n = 8)	3.11 ± 0.17 (n = 8)	3.58 ± 0.19 (n = 6)
Hepatic triglycerides (mg/g total lipids)	96.61 ± 7.51 (n = 8)	107.98 ± 10.66 (n = 8)	113.75 ± 4.56 (n = 8)	85.22 ± 19.75 (n = 6)
Cholesterol (mg/g total lipids)	39.49 ± 2.99 (n = 8)	45.31 ± 3.85 (n = 4)	84.49 ± 5.03 (n = 8)^{a,b,c}	47.24 ± 9.09 (n = 6)
HDL cholesterol (mg/g total lipids)	1.64 ± 0.12 (n = 8)	2.61 ± 0.20 (n = 8)^{e}	2.90 ± 0.31 (n = 8)^{a,c}	1.91 ± 0.36 (n = 6)

Significant differences ($P \leq 0.05$), according to one-way ANOVA followed by Student-Newman-Keuls post-hoc testing, are identified by the superscript letters as follows: ^{a}III versus I; ^{b}III versus II; ^{c}III versus IV; ^{d}IV versus I; ^{e}II versus I.

4. Discussion

4.1. General Aspects. In the present work the action of an *A. blazei* extract pretreatment on the paracetamol injury was examined not only in terms of the classical indicators, such as levels of hepatic enzymes in the plasma, levels of antioxidant enzymes in the liver and brain tissues, and oxidative stress indicators [14], but also in terms of some functional and metabolic parameters. The classical experimental protocol of the paracetamol injury model was followed in which several indicators are evaluated at 48 hours after oral paracetamol administration [14]. The *A. blazei* pretreatment was able to modify several aspects of the responses of the rat to paracetamol. One of these aspects was the appearance of paracetamol in the plasma, for which a shift of approximately one hour in the time for maximal concentration was found. It is difficult to infer the causes of this phenomenon from the available data. Important for the interpretation of the results, however, is the question whether the shift in the time for maximal concentration could be responsible for the effects of the *A. blazei* extract. The peak concentrations in nontreated and *A. blazei* pretreated rats were the same and the decreases in the plasma paracetamol concentration during the first hour after the time at which the peak concentrations were observed were also approximately the same. This leads to the conclusion that the *A. blazei* pretreatment did not affect the hepatic biotransformation of paracetamol, which is central to its toxicity. In principle at least, it can be hypothesized that the *A. blazei* extract might have delayed intestinal absorption of paracetamol, but this is a question that remains to be elucidated by future work. On the other hand, one hour delay represents a minimal fraction of the 48 hours period after which the injury parameters were measured. It is, thus, unlikely that the phenomenon could have a significant influence on the values of the various parameters that were measured.

In the following sections, the action of *A. blazei* will be discussed in terms of both the classical indicators for tissue damage as well as in terms of the functional and metabolic parameters.

4.2. Classical Parameters of Hepatoprotection. The most pronounced oxidative damage caused by paracetamol, which also plays a likely role in membrane damage, seems to be lipid peroxidation, which is revealed by the high TBARS levels found in both liver and brain. The aqueous extract of *A. blazei* was able to maintain normal levels of lipid peroxidation (TBARS) in both tissues. The phenomenon can, in principle at least, be attributed to the free-radical scavenging ability of the *A. blazei* extract [40]. This free-radical scavenging ability can be attributed largely to its high content in phenolic compounds because the three phenolics that have been identified in *A. blazei*, gallic acid, syringic acid,

and pyrogallol have been also demonstrated to possess high antioxidant activities [41]. Furthermore, their pronounced hydrophilic character makes it highly probable that they are effectively present in the aqueous *A. blazei* extract used in the present study. Phenolic antioxidants act as scavengers of radicals, and sometimes as metal chelators, acting both in the initiation step and the propagation of oxidation. Intermediate products formed by the action of these antioxidants are relatively stable due to resonance of the aromatic ring by these substances [42]. Antioxidant action can also be exerted by polysaccharides, whose presence in the *A. blazei* extract is well documented [43, 44]. In line with this proposition it must be mentioned that the hepatoprotective action of partially purified fungal polysaccharides has been recently demonstrated [45, 46]. Further components of *A. blazei* with antioxidant activity might also be oligopeptides. In fact an oligopeptide from *A. blazei*, rich in Pro, Lys, and Phe and possessing antioxidant activity has been described [47].

The diminution of the GSH levels in both liver and brain upon paracetamol injection was not very pronounced, at least not at 48 hours after the injury. Even so the effect of the *A. blazei* treatment was in the direction of normalization in both liver and brain. In addition to the effects on the TBARS levels, another prominent effect of the *A. blazei* extract pretreatment against the paracetamol injury was that on the membrane integrity. This was evidenced by the hepatic enzyme levels in the plasma (AST, ALT, LDH, and ALP), whose increases due to paracetamol injury were in all cases markedly prevented by the *A. blazei* treatment.

In relation to the activities of antioxidant enzymes CAT, SOD, GPx, and GR, the paracetamol injury diminished only the first two in the liver. In the brain paracetamol diminished all these activities, though to lesser extents. Interpretation of the results is not easy because it is difficult to correlate the pronounced diminution of the hepatic CAT levels caused by the paracetamol injury with the absence of a significant increase in the hepatic ROS levels. The *A. blazei* pretreatment partially prevented the consequences of paracetamol injury, most notably for the hepatic CAT levels and the brain SOD and GR levels, which were preserved close to normal. It is possible that this phenomenon was at least partly caused by adenosine or other purinergic agents which have been demonstrated to be important components of *A. blazei* [13]. Nucleosides and nucleotides are purinergic agents and purinergic effects of an *A. blazei* extract have been recently demonstrated to occur in the rat liver [13]. Adenosine, but possibly also other activators of A_1 purinergic receptors, confers cytoprotection in the cardiovascular and central nervous systems by activating cell surface adenosine receptors [48, 49]. Activation of these receptors, in turn, is postulated to activate antioxidant enzymes via protein kinase C phosphorylation of the enzymes or of intermediates that promote activation [48].

It should be remarked that in respect to the parameters discussed above, the action of the *A. blazei* extract pretreatment on the paracetamol injury is similar to that reported for the injury induced by carbon tetrachloride [50]. The same action was observed in patients with hepatitis B [11]. Also, extracts of other mushroom species have been shown to act similarly to the *A. blazei* extract on paracetamol injury, namely, *Lentinula edodes*, *Grifola frondosa* and *Tricholoma labayense* [14]. It must be remarked that other species, namely, *Volvariella volvacea, Flammulina velutipes, Auricularia auricular,* and *Tremella fuciformis,* failed to exert significant hepatoprotective actions [13] so that the hepatoprotective ability is not a universal characteristic among mushrooms.

4.3. Functional and Metabolic Parameters. Gluconeogenesis impairment was one of the most important consequences of paracetamol injury. The phenomenon reported in the present work bears probably little or no relation to the same phenomenon reported earlier [20] in the isolated perfused rat liver under constant paracetamol infusion. Under these conditions paracetamol inhibits gluconeogenesis by virtue of its inhibitory action on oxidative phosphorylation, which is a reversible effect. As long as paracetamol is present in the circulation it will obviously be exerting this action, but in the present work the livers were perfused 48 hours after paracetamol administration and the small amount of drug still present was rapidly washed out into the perfusion fluid. Consequently, the metabolic effects of paracetamol, including gluconeogenesis diminution, were most likely the consequence of the injury induced by the drug. In this respect the signs of inflammation that were observed, especially the presence of neutrophils and monocytes in addition to the inflammatory foci around the hepatic parenchyma, are highly significant. Inflammatory states are frequently, if not always, associated with decreased rates of gluconeogenesis. For example, diminished gluconeogenesis was found in livers from arthritic rats [51], in livers from rats poisoned with the venom of *Loxosceles intermedia* [52], and also in livers from rats injected with the inflammatory cytokines [53]. It seems thus reasonable to conclude that impairment of gluconeogenesis by paracetamol is closely associated with the inflammatory state. The *A. blazei* pretreatment was unable to prevent both. The pretreatment was also not effective in preventing the increased ammonia production induced by paracetamol, a parameter that indicates some kind of impairment of ammonia detoxification. The only normalizing effect of *A. blazei* on the metabolic effects of paracetamol was that on lactate production. These observations, in addition to the finding that the extract itself caused a diminished urea production, indicates some degree of interference with the hepatic metabolism. This interference must be the result of medium- and long-term effects because the components of the extract able to exert short-term effects as well, such as nucleosides and nucleotides [13], are no longer present in the isolated perfused liver. It should be added that it is unlikely that the gluconeogenesis impairment caused by paracetamol is a consequence of the loss of enzymes such as alanine aminotransferase. Lactate and pyruvate productions were increased by the paracetamol injury and alanine transformation into these products occurs necessarily via alanine amino-transferase. Furthermore, the *A. blazei* treatment reduced significantly the paracetamol-induced release of ALT and other enzymes, without preventing the negative effect on gluconeogenesis.

Pretreatment with the *A. blazei* aqueous extract had also little influence on the paracetamol diminished hepatic glycogen levels. The latter is a well-known consequence of

paracetamol overdosing [39], which is likely to be related to the gluconeogenesis impairment if one considers that a significant fraction of the hepatic glycogen synthesis depends on this metabolic pathway [54]. Singularly, however, the *A. blazei* pretreatment was able to prevent the increased levels of hepatic cholesterol induced by paracetamol. The data available so far do not allow any mechanistic interpretation for this phenomenon, but this observation indicates again that the *A. blazei* extract is able to exert metabolic effects. It is worth to mention that the hydroalcoholic extract of the flowers of *Calotropis procera* was also found able to normalize the cholesterol levels in rats of paracetamol injured rats [55].

5. Conclusions

If one considers only the classical indicators for tissue damage, the results that were obtained in the present study can be regarded as a positive indication that the *A. blazei* extract is able to provide a reasonable degree of protection against the paracetamol injury in both the hepatic and brain tissues. With respect to the functional and metabolic parameters of the liver, however, the extract provided little or no protection against most alterations caused by paracetamol. This includes microscopic morphological characteristics and the especially important functional parameter gluconeogenesis. This and other functional parameters, however, are usually not measured when hepatoprotection is examined. Considering our results and the long list of extracts and substances that are said to have hepatoprotective effects, however, it would be useful to incorporate evaluations of functional parameters into the experimental protocols of studies aiming to attribute or refute effective hepatoprotective actions.

Conflict of Interests

The authors state that they have no conflict of interests concerning the present paper.

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Review Article

Fructation *In Vivo*: Detrimental and Protective Effects of Fructose

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There is compelling evidence that long-term intake of excessive fructose can have deleterious side effects in different experimental models. However, the role of fructose *in vivo* remains controversial, since acute temporary application of fructose is found to protect yeast as well as animal tissues against exogenous oxidative stress. This review suggests the involvement of reactive carbonyl and oxygen species in both the cytotoxic and defensive effects of fructose. Potential mechanisms of the generation of reactive species by fructose in the nonenzymatic reactions, their implication in the detrimental and protective effects of fructose are discussed.

1. Introduction

Over the past decade, considerable scientific debate and controversy have arisen regarding the biological role of the reducing monosaccharides, and fructose in particular [1, 2]. Since many nutritionists believe that fructose is safer and healthier than glucose, fructose often is advocated as a glucose substitute by diabetes mellitus patients and a preferred sweetener for different population groups. At the same time, numerous epidemiological, clinical, and experimental studies demonstrate strong positive relationship between the intake of fructose and the development of metabolic disturbances [3–16]. Although consumption of fructose may have the adverse side effects and some authors state that there are no data showing a protective effect of fructose [3], it should also be mentioned that acute temporary application of fructose is found to be beneficial under some conditions [17–23].

Potential mechanisms underlying both detrimental and protective effects of fructose are under debate. Nonenzymatic reactions of fructose and higher production of reactive carbonyls (RCS) and oxygen species (ROS) compared with glucose are believed to be causative in negative effects of fructose [24–26]. However, ROS and RCS are found to play a dual role *in vivo*, which appears to be dose and time dependent [23, 27–32]. Therefore, we suggest the involvement of reactive species in both the cytotoxic and defensive effects of fructose. This review examines some of the potential mechanisms of ROS and RCS generation by the nonenzymatic reactions of fructose, their implication in the detrimental and defensive effects of fructose *in vivo*, and some of the differences between the long-term and short-term applications of fructose.

2. Involvement of Fructose in the Maillard Chemistry

The nonenzymatic reaction between amino acids and reducing monosaccharides was first described by Maillard a century ago [33, 34]. 40 years later, the Maillard reaction was recognized as one of the main reasons for the occurrence of the nonenzymatic food browning demonstrating an importance in food science [35, 36]. In late 1960s, the products of nonenzymatic glycosylation similar to the products of food browning were detected in human organism [37, 38]. It took several decades to realize the physiological significance of the reaction described by Maillard, which received renewed attention in biochemistry and medicine. The nonenzymatic glycosylation has been named "glycation" in order to differentiate it from the enzymatic glycosylation, an important posttranslation modification of proteins [39]. In 1980s, Monnier and Cerami postulated that glycation had a causative role in aging and age-related pathologies [40, 41]. Today, their theory called the "glycation hypothesis of aging"

is at the origin of the growing interest in the field of *in vivo* glycation, aging, and age-related diseases.

Glycation is a process in which various compounds, including RCS and advanced glycation end-products (AGEs), are produced [36, 42–45]. An increase in the RCS and AGEs steady-state levels may result in so-called carbonyl stress. The concept of "carbonyl stress" was introduced by Miyata and colleagues in late 1990s [46]. The authors have defined carbonyl stress as a situation "resulting from either increased oxidation of carbohydrates and lipids (oxidative stress) or inadequate detoxification or inactivation of reactive carbonyl compounds derived from both carbohydrates and lipids by oxidative and non-oxidative chemistry." RCS are mainly known for their damaging effects. At the molecular level, RCS are found to modify the structure of proteins, nucleic acids, lipids, and carbohydrates. As a consequence, the loss of functions and even viability can occur at the cellular and organismal levels. These harmful effects of RCS are mainly linked to the initiation of glycation [36, 43, 47]. Therefore, a vicious cycle can be created *in vivo* when RCS serve as either the initiators or products of glycation.

It should be noted that reactive carbonyls are commonly generated *in vivo* as metabolic products [36, 44, 45, 48]. For example, oxidation of such amino acids as threonine and glycine can lead to RCS formation under physiological conditions [49]. Different RCS can be generated *in vivo* by activated human phagocytes. It has been found that stimulated neutrophils employed the myeloperoxidase-H_2O_2-chloride system to produce α-hydroxy and α,β-unsaturated aldehydes from hydroxy amino acids in high yield [49].

Besides highly reactive RCS, carbohydrates are important glycating agents. In general, RCS may demonstrate 20,000-fold higher reactivity than some reducing monosaccharides [48]; however the latter are more abundant intra- and extracellular glycation agents. The contribution of carbohydrates to nonenzymatic processes has been extensively investigated over few last decades. This may be attributed to either beneficial or detrimental effects of reducing carbohydrates *in vivo*, and most studies in the field of glycation are focused on glucose (glucation). Fructose is another reducing monosaccharide, a common component of honey, fruit juice concentrates, table sugar, and high-fructose corn syrup. It has been excessively consumed in human diets over the last decades, despite the evidence implicating fructose in the development of metabolic and other disorders [3–6]. However, glycation by fructose (fructation) has not been as thoroughly investigated as that of glucose.

The initial step of fructation is the covalent interaction between free carbonyl group of open-chain fructose and amino group of biomolecule, producing the Schiff base (Figure 1). The latter is an unstable compound that can be subjected to further isomerization (Heyns rearrangement) and form more stable Heyns adducts. The Heyns compounds as well as Amadori products derived from glucation are known as "early glycation products" or "fructosamines." The fructose moiety of the Heyns products can undergo enolization followed by dehydration, oxidation, and/or fragmentation reactions, consequently producing a variety of RCS [36, 50–52].

RCS can also be formed due to enzymatic reactions of reducing carbohydrates (e.g., glucose or fructose). For example, polyol pathway may be associated with the production of glyoxal, methylglyoxal, glucosone, and 3-deoxyglucosone [49]. Effective steady-state concentration of such RCS metabolites as acetaldehyde, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate is typically low in the cell because of their rapid utilization by the next step of the pathway [49]. However, the concentration of such by-products of glycolysis, polyol pathway, or enzymatic oxidation of ketone bodies as glyoxal, methylglyoxal, glucosone, and 3-deoxyglucosone is not so tightly controlled [48, 49]. In general, unlike enzymatic reactions, nonenzymatic processes are not tightly controlled, and therefore they can be harmful.

Oxidation reactions and ROS have been shown to be involved and frequently accelerate the fructation, glucation, and other nonenzymatic processes [24, 36]. In order to reflect the interplay between glycation and oxidative steps the "glycoxidation" term has been introduced [53]. Figure 1 shows the mechanism of fructation followed by the generation of reactive di- and tricarbonyls as well as such ROS as superoxide, hydrogen peroxide, and hydroxyl radical. Slow oxidative degradation of monosaccharides under physiological conditions also leads to the formation of α-dicarbonyls and some ROS. This process has been called monosaccharide autoxidation or the Wolff pathway [44, 54, 55]. Figure 2 demonstrates the mechanism of fructose autoxidation. Like other early glycation products, the Heyns compounds may undergo autoxidation (Figure 3). This process leading to the formation of RCS and ROS has been called the Hodge pathway [35, 56, 57]. In addition, there is an evidence for the fragmentation of the Schiff base that results in RCS and ROS generation (Figure 4). The series of reaction pathways in glycation established the Schiff base fragmentation to RCS and ROS now collectively called the Namiki pathway [44, 51, 58, 59]. Thus, some stages of glycoxidation demonstrate a strong relationship between carbonyl and oxidative stress (Figure 5). Interestingly, some compounds were simultaneously identified as the intermediates or end-products of glycoxidation and lipid peroxidation that confirms an interplay between both the nonenzymatic processes [49].

In the late stage of glycation, the reactive carbonyls and the Heyns compounds again interact with free amino, sulfhydryl, and guanidine functional groups of intracellular or extracellular biomolecules like proteins, nucleic acids, and aminophospholipids, resulting in their nonenzymatic, irreversible modification and formation of a variety of adducts and crosslinks collectively named advanced glycation end products (AGEs) [44, 45]. Therefore, the Heyns products and RCS formed during fructation are believed to be important precursors of nonenzymatic adduct formation in biological systems. In general, AGEs are poorly degraded complexes (Figure 6) accumulation of which increases with aging. They were detected in a variety of human tissues and serve as biomarkers of aging and age-related disorders [36]. Interestingly, the comparison of nutrition and plasma AGEs in vegetarian and omnivorous groups showed that the higher

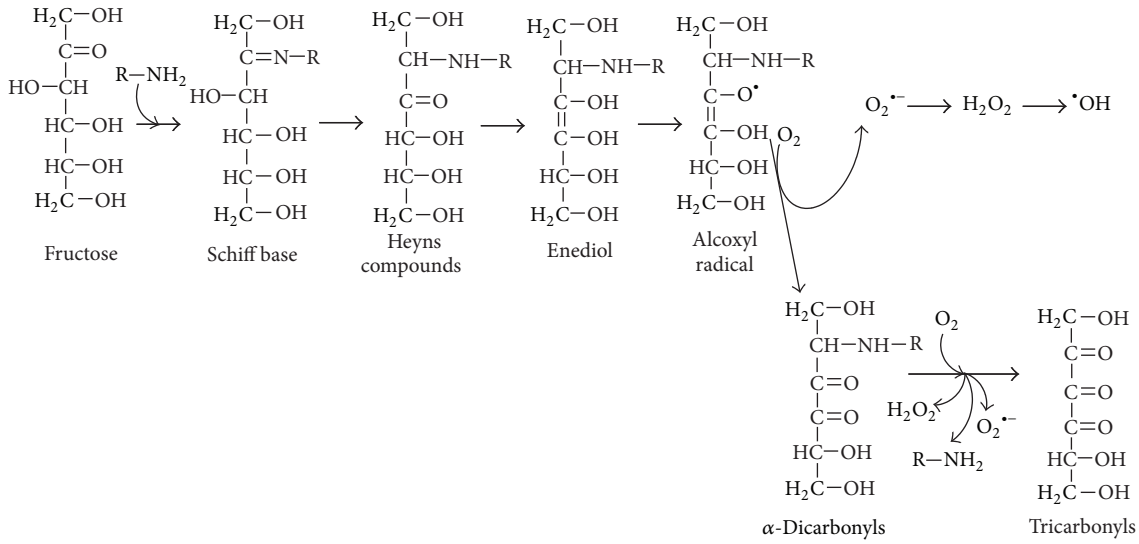

Figure 1: Suggested mechanism for the production of reactive carbonyl and oxygen species by the fructation.

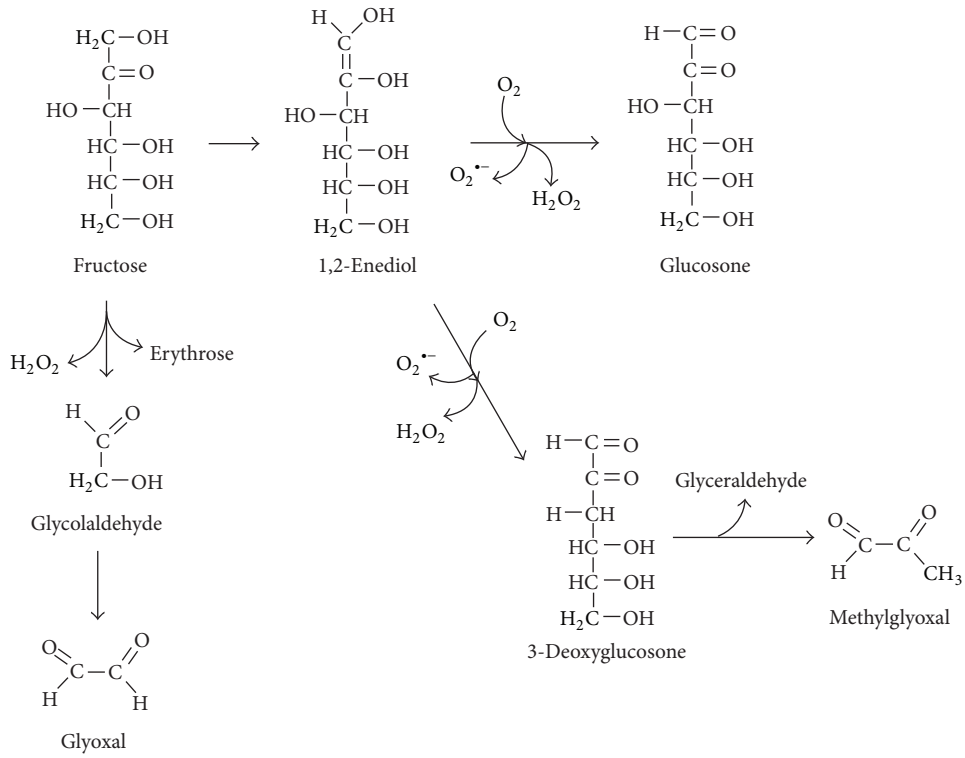

Figure 2: Fructose autoxidation (Wolff pathway).

intake of fructose in alternative nutrition of healthy subjects may cause an increase of AGE levels [60].

It should be noted that AGEs may undergo covalent interactions with biomolecules giving more complex cross-links. In addition, AGEs are efficient *in vivo* sources of RCS and ROS [44, 51, 61–63]. Like free-radical chain reaction, glycation is characterized by unpredictable direction and a wide variety of intermediates and end-products. That is why the term "Maillard chemistry" is widely used to describe the complicity of glycation [36, 43–45, 49].

3. Adverse Side Effects of Long-Term Consumption of Fructose

Fructose is commonly used as a sweetener and its intake has quadrupled since the early 1900s, in part because of

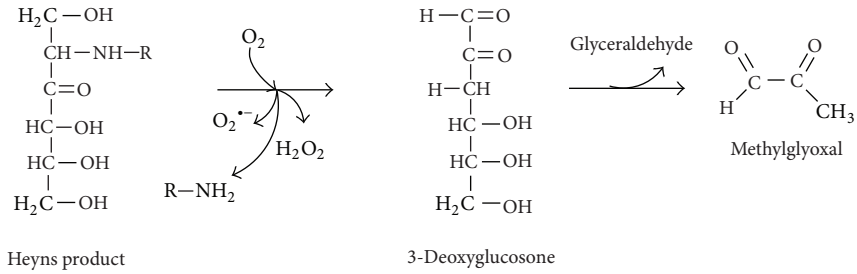

Figure 3: Autoxidation of Heyns compounds (Hodge pathway).

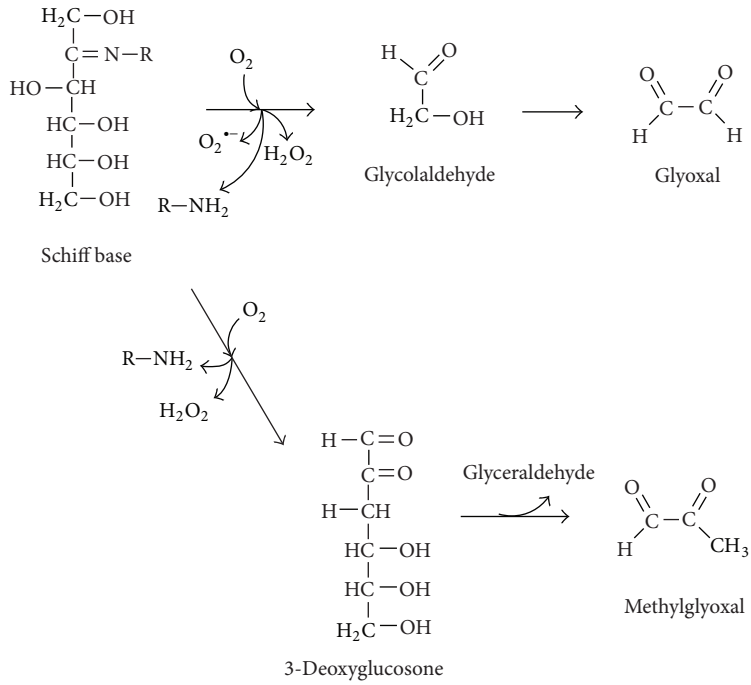

Figure 4: Oxidative fragmentation of Schiff base (Namiki pathway).

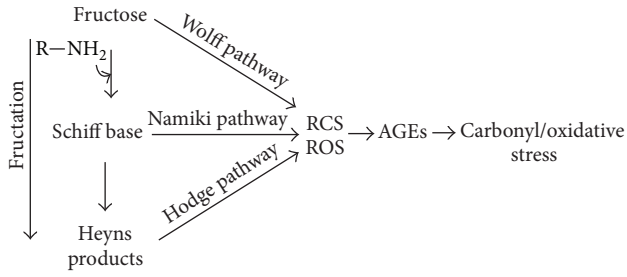

Figure 5: Formation of reactive species by the fructation leading to carbonyl/oxidative stress.

the introduction of high-fructose corn syrup [1]. This phenomenon parallels the development and progression of such disorders as obesity, type 2 diabetes mellitus and its complications, cardiovascular and neurodegenerative diseases, hypertension, and gout, liver, and kidney disease [1, 3, 7–16]. Experimental studies on animals have shown that chronic intake of excessive fructose can induce most features of the metabolic syndrome, including hypertriglyceridemia, fatty liver, nonalcoholic steatohepatitis, glucose intolerance, hyperglycemia, abdominal obesity, elevated blood pressure, microvascular disease, endothelial dysfunction, inflammation, hyperuricemia, glomerular hypertension, and renal injury [9, 16, 64–66].

Although the consumption of large amounts of dietary fructose can rapidly induce insulin resistance, most nutritionists believe that fructose is safer and healthier than glucose; therefore, fructose is advocated as a preferred sweetener, particularly for diabetes mellitus patients. Chronic hyperglycemia that in part can result from glucose intolerance induced by long-term consumption of fructose is a major inducer of vascular complications in diabetes (e.g., heart disease, stroke, blindness, and end-stage renal failure) which are responsible for disabilities and high mortality rates in patients with diabetes. The increased production of ROS, RCS, and AGEs as a result of glycoxidation is most preferable among the various mechanisms, which are supposed to be involved in vascular complications in diabetes. In general,

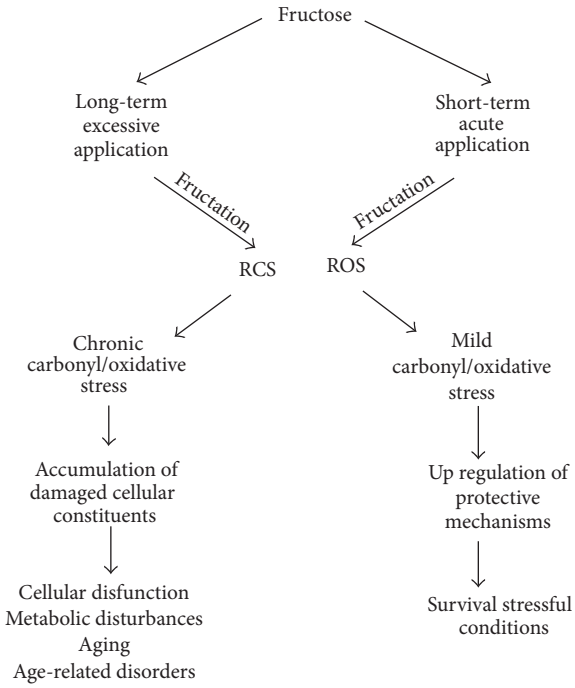

Figure 6: Involvement of reactive oxygen and carbonyl species in cytotoxic and defensive effects of fructose.

the enhanced levels of glycoxidation products can explain the detrimental effects of fructose due to its long-term application in different experimental models [67–71].

To compare glucose and fructose involvement in the generation of glycoxidation products, recently we used baker's yeast as a model and found higher level of carbonyl/oxidative stress markers, which were correlated with a higher aging rate of fructose-grown compared with glucose-grown yeast at stationary phase (long-term model) [25, 26]. We suggested that fructose rather than glucose is more extensively involved in glycoxidation *in vivo*, yeast aging, and development of carbonyl/oxidative stress. O'Brien with colleagues found that excessive fructose intake in animal models caused tissue damage associated with carbonyl/oxidative stress [72, 73]. *In vitro* experiments also demonstrated that fructose produced greater amounts of ROS and RCS than did glucose [24, 68, 69, 74].

At the first glance, from the point of view of basic organic chemistry it may seem surprising, since due to a greater electrophilicity and accessibility of the carbonyl group of aldoses (e.g., glucose), their reactivity is believed to be higher than that of respective ketoses (e.g., fructose). There is some information confirming higher reactivity of glucose versus fructose in nonenzymatic processes *in vitro* [75–77], while the opposite is reported in numerous *in vitro* and *in vivo* studies [24, 74, 78–80]. Possible explanation is that glucose is less reactive due to formation of very stable ring structures in aqueous solutions (glucopyranose and glucofuranose) which retards its reactivity. Generally, glucose is the least reactive monosaccharide and this characteristic can be considered to the emergence of glucose as the primary metabolic fuel [36, 81, 82]. Fructose also forms both pyranose and furanose structures [83] but exists to a greater extent in the open-chain active form than does glucose. The proportion of acyclic forms of glucose and fructose in aqueous solution accounts for 0.001–0.002% and 0.7%, respectively [48, 84]. Thus fructose is a more potent glycoxidation agent as compared with glucose that can explain its detrimental effects.

4. Short-Term Application of Fructose Protects against Oxidative Stress

Although a long-term consumption of excessive fructose may have adverse side effects, its acute temporary ingestion can be beneficial under some circumstances. For example, short-term application of fructose has been found to protect astroglial C6 cells against peroxide-induced stress [21]. In contrast to glucose, fructose inhibited apoptosis induced by reoxygenation in rat hepatocytes by decreasing the level of ROS [17]. Fructose has also been found to defend rat hepatocytes against exogenous oxidative stress [18, 20]. It has been demonstrated that fructose and its phosphorylated derivatives such as fructose-1,6-bisphosphate had significantly higher antioxidant capacities against ROS than other carbohydrates [22]. Based on these phenomena, it was suggested that acute infusion or ingestion of fructose could be of benefit in the cytoprotective therapy of disorders related to oxidative stress [21]. According to homeostasis theory, the steady-state concentration of oxidants as well as antioxidants is maintained at the limited range [85]. That is why antioxidant therapy is generally found to be ineffective [30, 31]. In contrast to strong antioxidants, fructose and its phosphorylated derivatives (e.g., fructose-1,6-bisphosphate) being important energy substrates are not "fought" by redox homeostatic mechanisms. The beneficial effects of fructose-1,6-bisphosphate have been documented in different tissues, including the heart, liver, kidney, brain, and small intestine [86–89]. The cytoprotective mechanisms underlying fructose-1,6-bisphosphate are believed to be involved in its intervention in the glycolytic pathway, as a metabolic regulator or substrate, as well as an agent modifying the ion permeability of cell membrane transporters.

Recently we have demonstrated that fructose-grown yeast at exponential phase (short-term model) exposed to hydrogen peroxide demonstrated higher survival compared to glucose-grown cells [23]. In this study, significantly higher total level of ROS was observed in fructose-grown than that in glucose-grown cells under control conditions (without H_2O_2). However, under peroxide-induced stress ROS amount significantly decreased in yeast grown on fructose, whereas it increased in glucose-grown cells, which was very consistent with the work of Spasojević et al. [21]. The authors demonstrated a significant increase in oxidative status of astroglial C6 cells under treatment with hydrogen peroxide in glucose medium, but it was not the case in a fructose-containing medium. At the same time, hydrogen peroxide led to a decrease of C6 cell viability in both media investigated; however, the survival was higher in fructose-containing medium. In accordance with another work by

Spasojević et al. [22], we demonstrated that hydrogen peroxide did not markedly change hydroxyl radical level in glucose-grown cells but it did decrease it significantly in fructose-grown cells [23].

An obvious question arises: what mechanism(s) is (are) responsible for the protective effect of fructose short-term application? Analysis of the literature data leads us to propose several mechanisms responsible for the defensive effect of fructose: (1) iron binding and prevention of the Fenton reaction [18, 21]; (2) stabilization of the glutathione pool in the cell [17]; (3) upregulation of the pentose phosphate pathway producing NADPH [22]; and (4) production of fructose-1,6-bisphosphate, the compound with cytoprotective and antioxidant mechanisms [86–89].

Our study extended the earlier findings with the involvement of SOD and catalase in the reduction of ROS level in fructose-grown yeast exposed to H_2O_2 [23]. It was shown that a reduced ROS level in fructose-grown cells was consistent with a broad peak of SOD and catalase activation by hydrogen peroxide, whereas cells grown on glucose demonstrated a sharp rise of the enzyme activities. We also found that fructose more markedly than glucose activated glyoxalases, the fundamental function of which is the metabolism of reactive α-dicarbonyl metabolites in most living organisms [43, 90–92].

These findings prompted us to propose additional explanation of fructose protective effect—a short-term application of fructose induces a mild carbonyl/oxidative stress-stimulating cellular defensive mechanisms responsible for cell survival under lethal stress [23]. The last mechanism can be posited from the *in vitro* and *in vivo* studies reporting that fructose is a much more potent glycoxidation agent, capable of producing greater amounts of RCS and ROS than glucose [21, 23–26, 68, 74]. Generally, ROS and RCS are found to play a dual role *in vivo*, which appears to be concentration dependent [23, 27–32]. At high concentrations, reactive species are potentially dangerous, as they can cause damage to cell constituents that, in turn, accompany aging and age-related disorders. In contrast, the beneficial effects of ROS and RCS occur at low concentrations and involve physiological roles in cellular signaling pathways, responses to environmental challenges, and so forth. It is also well documented that mild stress caused by low doses of reactive species can result in the acquisition of cellular resistance to lethal stress [28, 93–96].

5. Conclusion

Considering the literature data it can be supposed that at long-term consumption of excessive fructose chronic increase in the levels of reactive species leads to the accumulation of damaged cellular constituents resulting in cellular disfunction, whereas short-term application of fructose provokes a mild stress, resulting in the acquisition of cellular cross-resistance to lethal stress (Figure 6). Therefore, we suggest the involvement of reactive species like RCS and ROS in both the cytotoxic and defensive effects of fructose. Thus, depending on conditions, fructose can play a dual role in the living cell. Long-term application of excessive fructose leads to glycoxidation, generation of ROS and RCS, and accumulation of damaged cellular constituents which is suggested to accompany the aging process, cellular dysfunction. and age-related disorders. In opposite, short-term application of fructose provokes a mild carbonyl/oxidative stress, resulting in the acquisition of cellular cross-resistance to lethal stress. Generally, a long-term consumption of excessive fructose is found to have adverse side effects; however acute temporary application of fructose can be beneficial under some pathophysiological conditions.

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Review Article

Molecular Basis of Cardioprotective Effect of Antioxidant Vitamins in Myocardial Infarction

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Acute myocardial infarction (AMI) is the leading cause of mortality worldwide. Major advances in the treatment of acute coronary syndromes and myocardial infarction, using cardiologic interventions, such as thrombolysis or percutaneous coronary angioplasty (PCA) have improved the clinical outcome of patients. Nevertheless, as a consequence of these procedures, the ischemic zone is reperfused, giving rise to a lethal reperfusion event accompanied by increased production of reactive oxygen species (oxidative stress). These reactive species attack biomolecules such as lipids, DNA, and proteins enhancing the previously established tissue damage, as well as triggering cell death pathways. Studies on animal models of AMI suggest that lethal reperfusion accounts for up to 50% of the final size of a myocardial infarct, a part of the damage likely to be prevented. Although a number of strategies have been aimed at to ameliorate lethal reperfusion injury, up to date the beneficial effects in clinical settings have been disappointing. The use of antioxidant vitamins could be a suitable strategy with this purpose. In this review, we propose a systematic approach to the molecular basis of the cardioprotective effect of antioxidant vitamins in myocardial ischemia-reperfusion injury that could offer a novel therapeutic opportunity against this oxidative tissue damage.

1. Introduction

Acute myocardial infarction (AMI) is the leading cause of mortality worldwide. In 2008 ischemic heart disease accounted for 7.25 million deaths worldwide (12.8%), according to the WHO. It is of relevance to consider not only its impact in mortality, but also the impairment in the life quality of patients surviving this vascular accident. During the last decades, therapies in use have shown a significant mortality reduction in myocardial infarction patients [1–4]. However, such beneficial effects are still of limited efficacy, and new therapies are currently being investigated. Systemic thrombolysis and percutaneous coronary angioplasty (PCA) have been used to recover the myocardial perfusion, with the latter being the most successful, as it allows to reestablish the blood flow in the cardiac zones affected by the occlusion of a branch of the coronary artery. Nevertheless, as a consequence of this procedure, the ischemic zone is reperfused, giving rise to an ischemia-reperfusion event that generates increased production of reactive oxygen species (oxidative stress) [5], thus enhancing the previously established tissue damage (lethal reperfusion), as these reactive species attack biomolecules such as lipids, DNA, and proteins and trigger cell death pathways [6]. Studies on animal models of AMI suggest that lethal reperfusion accounts for up to 50% of the final size of a myocardial infarct, a part of the damage likely to be prevented [7]. Although a number of strategies have been aimed at to ameliorate lethal reperfusion injury, up to date the beneficial effects in clinical settings have been disappointing. The use of antioxidant vitamins could be a suitable strategy with this purpose, but oral administration does not allow reaching the plasma levels required to counteract the effects of oxidative stress [8]. Alternatively, short episodes of ischemia before total reperfusion such as short balloon inflations before final reperfusion during coronary angioplasty may have protective effects [9]. In a rabbit model, the administration of ascorbate aggravates damage, likely through the abrogation of the endogenous enzymatic antioxidant response triggered by short episodes of ischemia [10]. Some protocols using intravenous antioxidant vitamins

have failed to achieve a significant amelioration of infarct size. Reinforcement of the antioxidant defense system should be expected to protect the myocardium against the reperfusion injury. Indeed, at present, no study with this purpose has used ascorbate in doses high enough to scavenge superoxide anion. Interestingly, even high doses reaching plasma vitamin C levels higher than 10 mmoles/L have been administered in other clinical settings [11]. This dose and higher ones proved to be remarkably safe in a recent meta-analysis [12]. In this review we propose a systematic approach to the molecular basis of antioxidant vitamin treatment against ischemia-reperfusion injury in myocardial infarction.

2. Role of Ischemia-Reperfusion in Myocardial Infarction

2.1. Oxidative Stress in Myocardial Ischemia-Reperfusion

2.1.1. Major Role of Oxidative Stress. Oxidative stress constitutes a unifying mechanism of injury of many types of disease processes; it occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems in the body so that the latter become overwhelmed [13]. ROS are a family of highly reactive species that are formed either enzymatically or nonenzymatically in mammalian cells and causing cell damage either directly or through behaving as intermediates in diverse cell signaling pathways. Antioxidant defenses can be divided into enzymatic and nonenzymatic. Enzymatic antioxidant defenses mainly include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and thioredoxin peroxidase, among others. Nonenzymatic antioxidant defenses include a variety of biological molecules, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), reduced glutathione (GSH), coenzyme Q10, cysteine, carotenoids, flavonoids, polyphenols, and other various exogenous antioxidants [14, 15]. Polyphenols act as antioxidants both through the prevention of damage from ROS and their iron chelating ability [16], further enhancing the *in vivo* antioxidant potential. The first line of cellular defense against oxidative injury in the heart as well as most tissues includes the antioxidant enzymes CAT, SOD, and GSH-Px [15]. There are many mechanisms through which antioxidants may act such as (1) scavenging reactive oxygen species or their precursors, (2) inhibiting the formation of ROS, (3) attenuating the catalysis of ROS generation via binding to metal ions, (4) enhancing endogenous antioxidant generation, and (5) reducing apoptotic cell death by upregulating the antideath gene Bcl-2 [14].

In normal physiological conditions, oxygen-free radical production is usually kept under homeostatic control by endogenous free radical scavengers known as antioxidants. However, during ischemia there is a loss of antioxidant enzyme function, together with leakage of antioxidant enzymes into the extracellular fluid. The levels of myeloperoxidase (MPO), a strong prooxidant enzyme, have a prognostic role in acute coronary syndromes. MPO levels were associated with no-reflow phenomenon or impaired myocardial microcirculation in STEMI patients. Neutrophil activation occurring in ischemia-reperfusion is one of the major determinants of vascular impairment in myocardial tissue, and MPO is just a marker of neutrophil activation [28]. On reperfusion, the enzymes are then washed out, further depleting the available control over free radical production, and as a result, the unbalanced burst of free radicals on reperfusion (respiratory burst) easily overwhelms the available counteractive enzymes so the control of ROS generation is lost [14].

In addition, AMI is a clinical model of oxidative stress by ischemia-reperfusion. Reactive oxygen species (ROS) are major initiators of myocardial damage during ischemia/reperfusion. Accordingly, AMI is usually initiated by myocardial ischemia due to coronary artery obstruction. In pathophysiological conditions, sources of ROS include the mitochondrial respiratory electron transport chain, xanthine oxidase activation due to ischemia-reperfusion, the respiratory burst associated with neutrophil activation, and arachidonic acid metabolism. Several studies have proposed the essential role of ROS in the pathogenesis of myocardial ischemia-reperfusion injury. ROS including hydrogen peroxide (H_2O_2), superoxide radical, hydroxyl radical (OH·), and peroxynitrite ($ONOO^-$) have been shown to increase upon reperfusion of the heart following ischemia [29]. An increase in the formation of ROS during ischemia-reperfusion was also reported by using the electron paramagnetic resonance technique. ROS seem to increase significantly after a few minutes of reperfusion, but its increase during ischemia alone is still controversial. On the basis of these changes it has been suggested that the increase of H_2O_2 production and other ROS during ischemia-reperfusion leads to lipid peroxidation and sulfhydryl group oxidation [30].

2.1.2. Pathophysiology of Myocardial Ischemic Injury. A series of biochemical and metabolic changes in myocardial tissue occur due to deprivation of oxygen and nutrient supply during ischemia. Consequently mitochondrial damage and ATP depletion impair myocardial contractile function [9]. Anaerobic glycolysis due to the absence of oxygen results in the accumulation of lactate and intracellular pH reduction (to <7.0). The latter activates the Na^+-H^+ ion exchanger, thus extruding protons from the cell in exchange for Na^+ entry. Furthermore the impaired function of (Na + K)-ATPase contributes to exacerbate the intracellular Na^+ and Ca^{2+} overload [31].

2.1.3. Pathophysiology of Myocardial Reperfusion Injury. The level of tissue oxygenation increases following restoration of blood flow, which is followed by a burst of ROS generation that leads to the syndrome of reperfusion injury [5]. Neutrophils are the primary source of ROS during reperfusion, although endothelial cells and cardiomyocytes can also generate this reactive species. Increased ROS production is mainly due to activation of xanthine oxidase in endothelial cells, mitochondrial electron transport chain reactions in cardiomyocytes, and NADPH oxidase in inflammatory cells [32]. Under these conditions, the enzymatic antioxidant

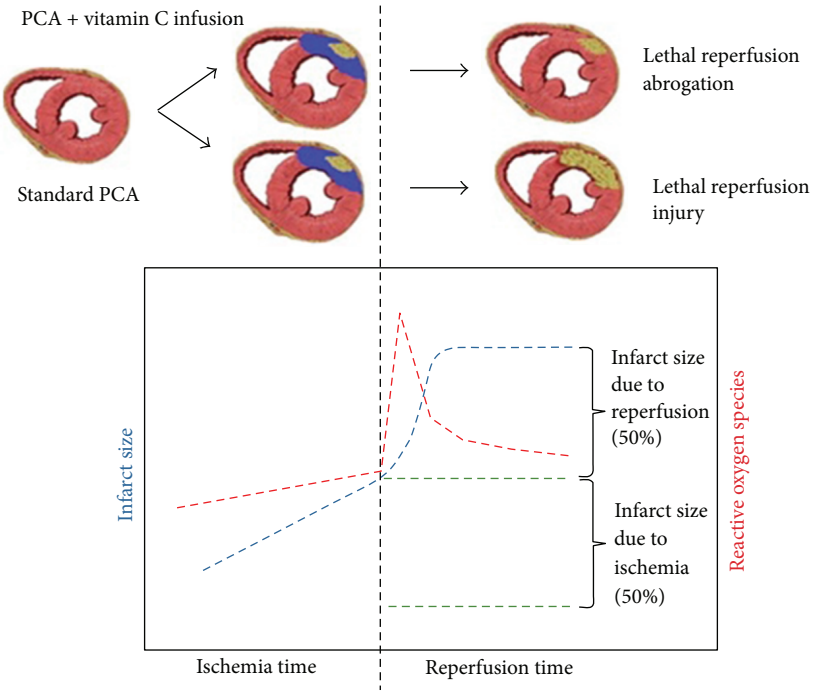

Figure 1: Schema representing the time course of the effects of ischemia reperfusion. Upon reperfusion there is an oxidative burst, which corresponds to a marked increase in infarct size. Counteracting this process could account for a decrease of up to 50% infarct size.

effect is relevant against the detrimental effects of ROS. Therefore, it should be expected that a reinforcement of the antioxidant defense system through ROS scavengers results in a cardioprotective effect during the myocardial reperfusion (Figure 1). After an ischemic episode of the myocardium, left ventricle remodeling is known to occur; although its underlying mechanism is multifactorial, ROS and inflammatory cytokines may cause cardiodepressive reaction [33–35]. It is of interest to remark that ROS also stimulate the production of inflammatory cytokines and, in turn, inflammatory cytokines stimulate ROS formation. In chronic stage, ROS and inflammatory cytokines activate the matrix metalloproteinases [36, 37], thereby eliciting degradation of collagens which may cause a slippage in myofibrillar alignment causing left ventricular dilatation [38].

The ischemia-reperfusion injury includes a series of events: (a) reperfusion arrhythmias, (b) microvascular damage, (c) myocardial stunning "reversible mechanical dysfunction," and (d) cell death, all of which may occur either together or separately [39]. There are two main hypotheses, namely, oxidative stress and Ca^{2+}-overload, which have been proposed to explain the pathogenesis of ischemia-reperfusion injury [40, 41]. Both these mechanisms are most likely related to each other but it is not known whether they operate simultaneously or if one precedes the other. With respect to this, oxidative stress, which is usually associated with increased formation of ROS, modifies phospholipids and proteins leading to lipid peroxidation and thiol groups oxidation; these changes are considered to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins [42]. Oxidative stress may result in cellular defects including a depression in the sarcolemmal Ca^{2+}-pump ATPase and (Na + K)-ATPase activities, changes leading to decreased Ca^{2+}-efflux and increased Ca^{2+}-influx, respectively [43]. Oxidative stress has also been reported to depress the sarcoplasmic reticulum Ca^{2+}-pump ATPase and thus inhibit Ca^{2+} sequestration from the cytoplasm in cardiomyocytes. These alterations were markedly reduced by antioxidants such as catalase and superoxide dismutase [44]. The depression in Ca^{2+}-regulatory mechanism by ROS ultimately results in intracellular Ca^{2+} ($[Ca^{2+}]_i$) overload and cell death. On the other hand, an increase in $[Ca^{2+}]_i$ during ischemia induces the conversion of xanthine dehydrogenase to xanthine oxidase and subsequently results in generating superoxide radicals [44].

2.2. Other Mediators of Myocardial Injury

2.2.1. Inflammation in Ischemia-Reperfusion. ROS generation could occur through several enzymatic reactions in cell types such as endothelial, inflammatory, and cardiomyocyte cells. Among these enzymatic sources, much attention has been placed on xanthine oxidase in endothelial cells, NADPH oxidase in inflammatory cells and the mitochondrial electron transport chain reaction in cardiomyocytes using either *in vivo* models of ischemia-reperfusion or cultured endothelial cells and cardiomyocytes after hypoxia-reoxygenation [32, 45, 46]. It has been proposed that a burst of ROS from endothelial cells, and cardiomyocytes during early reperfusion can influence nearby neutrophils, setting up a local cycle of amplified cellular response through released inflammatory mediators.

Furthermore, neutrophils become sensitized (primed) to activating factors, such as chemotactic cytokines, after they adhere to the endothelium, and thus generate much greater quantities of ROS. Specifically, the chemokine interleukin-8 appears to have a fundamental role in regulating neutrophil localisation in ischaemic myocardium. In mice, CXCL2, the homologue of human interleukin-8, is upregulated in reperfused myocardium [47]. The chemokine response in ischaemic tissues may be induced by various factors, including ROS, cytokines (e.g., tumour necrosis factor (TNF)-α), the complement system, and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation, a major proinflammatory transcription factor [48]. After the initial burst of ROS at the onset of reperfusion, later events such as transendothelial migration of neutrophils and macrophages might participate in delayed ROS generation during reperfusion [49, 50]. Activated neutrophils produce superoxide as a cytotoxic agent as part of the respiratory burst via the action of membrane-bound NADPH oxidase on molecular oxygen. Despite the fact that superoxide anion per se is not a potent oxidant, its interaction with nitric oxide (NO) can lead to the powerful oxidant $ONOO^-$. In addition it should be mentioned that transition metal ions, such as iron, could give rise to the very harmful Fenton and Haber-Weiss reactions. Even small amounts of intracellular nonbound iron (labile iron pool) may interact with superoxide leading to the formation of extremely reactive hydroxyl radical. Also, in the presence of iron the antioxidant vitamins may act as prooxidants [51]. Neutrophils also produce the free radical NO that can react with superoxide to produce $ONOO^-$, a powerful oxidant, which may decompose to form OH·. Prospective epidemiological studies have shown that serum levels of C-reactive protein (CRP), a biomarker of inflammation, are a strong predictor of cardiovascular ischaemia-reperfusion injury cycle events, such as myocardial infarction, postoperative atrial fibrillation and stroke [52]. Several studies revealed an independent association of high plasma CRP levels with adverse prognosis in acute myocardial infarction patients. Interestingly, preconditioning was found to inhibit postischaemic CRP increases in a rat model of acute myocardial infarction [53].

2.2.2. Effect of pH on Cardiomyocyte Function. A decreased intracellular pH during ischemia is rapidly restored to physiological pH by the washout of lactate during reperfusion. It is of interest to note that pH shift contributes to the cardiomyocyte death of lethal myocardial reperfusion injury [54] by permitting mitochondrial permeability transition pore (mPTP) opening and cardiomyocyte rigor hypercontracture in the first few minutes of reperfusion. Also, it has been described that acidosis protects against lethal anoxic injury and that a rapid return from acidotic to physiologic pH significantly contributes to reperfusion injury to cardiac myocytes, a "pH paradox" [55].

2.2.3. Intracellular Ca^{2+} Overload . Dysregulation of Ca^{2+} homeostasis has long been implicated to play an important role in cell injury. Pathological Ca^{2+} overload and calcification are frequently features of tissue ischemia and infarction, and increased Ca^{2+} activates a number of phosphatases, proteases, and nucleases [56]. The effects of calcium overload in acute myocardial ischemia are due to disruption of the plasma membrane, oxidative stress-induced damage to the sarcoplasmic reticulum, and mitochondrial reenergization. Mitochondrial re-energization allows the recovery of the mitochondrial membrane potential that drives the entry of Ca^{2+} into mitochondria via the mitochondrial Ca^{2+} uniporter and subsequently induces the opening of the mPTP [57]. Ca^{2+} release from the endoplasmic reticulum may flood the cytosol with free Ca^{2+}, possibly leading to activation of degradative processes and dysfunction of other organelles, particularly mitochondria [58]. In addition, calcium overload besides other detrimental effects increases the arrhythmic risk by provoking afterdepolarizations in cardiac cells. Recent evidence suggests that blockade of calcium current was highly effective in suppression of early afterdepolarizations [59].

2.2.4. The Role of mPTP in Myocardial Ischemia. Many chemicals and radicals are inducers that promote the mPTP opening, thus decreasing the threshold amount of Ca^{2+} needed in that process [56]. Opening of mPTP results in mitochondrial membrane depolarization and uncoupling of oxidative phosphorylation, leading to ATP depletion and cell death [60, 61]. In has been shown that, in settings of acute myocardial ischemia reperfusion injury, the mPTP remains closed during ischemia and only opens at reperfusion in response to mitochondrial Ca^{2+} and phosphate overload, oxidative stress and relative ATP depletion, and rapid pH correction [62].

2.3. Cell Death Signaling Pathways: Apoptosis, Necrosis, and Autophagy. In AMI, ROS are generated in the ischemic myocardium especially after reperfusion. ROS directly injure the cell membrane and cause cell death [6]. However, ROS also stimulate signal transduction to elaborate inflammatory cytokines, for example, (TNF)-α, interleukin (IL)-1β, and -6, in the ischemic region and surrounding myocardium as a host reaction. Inflammatory cytokines also regulate cell survival and cell death in the chain reaction with ROS. Apoptosis or programmed cell death is a distinct form of destruction of the cell which is associated with synthesis of enzymes that degrade and fragment its own DNA. Briefly, the signal pathway of apoptosis involves the stimulation of cell membrane death receptors (Fas) which leads to the activation of caspases (aspartate-specific proteases), protein cleavage, DNA fragmentation, and cell death. Several studies have shown that myocardial ischemia-reperfusion is associated with an increase in apoptotic cells [63]. However, the exact mechanisms underlying the induction of this apoptotic process and the long-term consequences of this process in myocardial ischemia-reperfusion are not completely understood. Exposure of cultured rat cardiomyocytes to lower doses of an exogenous ROS-generating system, such as H_2O_2 and superoxide anion, caused release of cytochrome c and activation of caspase-3 and triggered apoptotic cell death [64]. Various studies suggest that release of ROS

from activated neutrophils [65] and macrophages [66, 67] may contribute to the early and progressively increasing apoptosis. A significant linear relationship between the number of apoptotic myocytes and transmigrated neutrophils, as well as macrophages, was also observed during early and prolonged reperfusion [68, 69]. Updated information suggests that ischemia followed by reperfusion significantly induces myocardial injury by an apoptotic death pathway. To understand potential signaling mechanisms involved in ROS-triggered apoptosis, recent reports have shown that intracellular Ca^{2+} overload and enhanced activity of the mitogen-activated protein kinase (MAPK) family during reperfusion can participate in induction of ROS-mediated apoptosis in addition to necrosis and eventually could be determinant of the infarct size [70].

Cell death was once viewed as unregulated. It is now clear that at least a portion of cell death is a regulated cell suicide process. This type of death can exhibit multiple morphologies. One of these, apoptosis, has long been recognized to be actively mediated, and many of its underlying mechanisms have been elucidated. Moreover, necrosis, the traditional example of unregulated cell death, is also regulated in some instances. Autophagy is usually a survival mechanism but can occur in association with increased ROS leading to cell death. Little is known, however, about how autophagic cells die [71]. Apoptosis, necrosis, and autophagy occur in cardiac myocytes during myocardial infarction, ischemia-reperfusion, and heart failure. Pharmacological and genetic inhibition of apoptosis and necrosis lessens infarct size and improves cardiac function in these disorders [72].

2.3.1. Apoptosis and Necrosis. Apoptosis is a highly controlled cell death process that is autonomously committed by both healthy and sublethally injured cells in response to physiological or pathological stimuli, including ischemia-reperfusion events. Necrotic cell death is a widely recognised property of ischemic cell death and is clinically diagnosed by documenting myocyte release of cytosolic constituents, such as creatine kinase MB, troponins, and other proteins. However, apoptosis has only been implicated in the pathogenesis of several acute and chronic conditions affecting the cardiovascular system in the last decade [73]. The loss of endothelial cells precedes and may predispose cardiomyocytes to undergo apoptosis [74], indicating that salvaging endothelial cells is of paramount importance. Whether myocyte apoptosis is initiated during ischemia but dependent on reperfusion or whether it is a feature of reperfusion injury requires further study. Reperfusion appears to accelerate apoptosis when compared with permanent occlusion [75]. In contrast to the modest, chronically elevated levels of cell death during heart failure, myocardial infarction is characterised by a large burst of cardiac myocyte death that is usually complete within 24 hours. Active caspases cleave vital substrates in the cell, such as actin, actinin, β-myosin heavy chain, myosin light chain, tropomyosin, and cardiac troponins, leading to cellular demise [76]. The "intrinsic" pathway utilises mitochondria to induce cell death by opening the mPTP or rupturing the outer mitochondrial membrane, both of which trigger the sudden and complete release of cytochrome c and other proteins from the intermembrane mitochondrial space into other cellular compartments. The "intrinsic" pathway is primarily activated in cardiac myocytes by cellular stimuli, such as hypoxia, ischemia-reperfusion, and oxidative stress, which perturb the mPTP and increase the permeability of the outer and inner mitochondrial membranes [77]. Once released, cytochrome c binds to the cytosolic protein Apaf1 and facilitates formation of the "apoptosome" complex, which results in caspase-9 activation that provokes caspase-3 activation [78]. Smac/DIABLO indirectly activates caspases by sequestering caspase-inhibitory proteins, while the mitochondrial release of endonuclease-G and apoptosis-inducing factor results in their translocation into the nucleus where they directly or indirectly facilitate DNA fragmentation [79]. The "extrinsic" apoptotic pathway involves the death-receptor Fas pathway. Binding of the transmembrane protein Fas to its cognate receptor induces receptor clustering and the formation of a death-inducing signalling complex. Cardiac overexpression of the Fas ligand results in accentuated apoptosis *in vitro*, whereas Lpr mice, which lack Fas, display less apoptosis and reduced infarct size in ischemia-reperfusion studies [80].

Apoptotic cell death can transition to necrosis during oxidative stress by two possible mechanisms. First, the inactivation of caspases due to oxidation of their active site thiol groups by oxidants or S-nitrosylation can lead to necrosis-like cell death in fatally damaged cells [81]. Second, a drop in ATP levels due to the failure of mitochondrial energy production by oxidants can cause apoptosis to change to necrosis [82]. In addition, it was recently found that the proapoptotic protein Bnip3 is associated with mitochondrial dysfunction and cell death. Bnip3 is also a potent inducer of autophagy in many cell types, including adult cardiac myocytes. Bnip3 overexpression induces selective removal of mitochondria in cardiac myocytes and triggers induction of autophagy independent of calcium, ROS generation, and mPTP opening [83]. Furthermore, it was recently reported that angiotensin II induces mitochondrial autophagy and biogenesis through mitochondrial ROS in the mouse heart [84].

2.3.2. Autophagy. In contrast to necrosis and apoptosis, autophagy is primarily a survival mechanism. Cellular oxidative stress and ROS have been reported to serve as important autophagic stimuli during periods of ischemia-reperfusion [85]. Autophagic degradation and removal of damaged oxidised proteins in response to low to moderate oxidative stress are reportedly beneficial for cells. Conversely, severe oxidative stress and increasing amounts of ROS may activate signalling pathways that lead to autophagy-induced cell death. Whether autophagy promotes cell survival or death depends upon the severity and degree of stress in the cellular environment [86]. During the initial period of ischemia, enzyme xanthine oxidase is formed, and substrates for xanthine oxidase (hypoxanthine and xanthine) accumulate. Upon reperfusion, the reintroduction of O_2 leads to xanthine oxidase-mediated superoxide generation due to the presence of xanthine and hypoxanthine [87].

During this period, ATP generation decreases and results in the phosphorylation of 5′adenosine monophosphate-activated protein kinase (AMPK), which leads to autophagosome formation through inhibition of mammalian target of rapamycin (mTOR) [88]. Meanwhile, ROS damage organelles and cytosolic proteins and cause mitochondrial lipid peroxidation, all of which exacerbate autophagy [89]. Additionally, antioxidant enzymes, such as CAT and SOD, are targeted by autophagosomes. This ultimately leads to the induction of cell death and is thus detrimental to tissue function. Furthermore, AMPK activity decreases during reperfusion, thus increasing autophagic death and upregulating beclin-1 [90]. Autophagy has been reported to be involved in cardioprotection against lethal ischemic injury; thus, repetitive ischemia by coronary stenosis or occlusion enhances autophagy and subsequent cardioprotection when compared with classical ischemia-reperfusion insult [91]. Aside from the key role that the chemokine monocyte chemoattractant protein-1 (MCP-1) plays in cardiac damage following ischemia, it also mediates autophagy through MCP-1-induced protein (MCPIP), a novel zinc-finger protein that has transcription factor-like activity [92]. MCPIP stimulates inducible NO synthase, translocation of the NADPH oxidase subunit phox47 from the cytoplasm to the membrane, ROS production, induction of endoplasmic reticulum stress markers HSP40, and autophagy, as indicated by beclin-1 induction, cleavage of microtubule-associated protein 1 light chain 3 and autophagolysosome formation, and apoptosis, respectively [93].

3. Role of Antioxidant Therapy in Myocardial Infarction

The therapeutic effects of vitamins C and E will be discussed in the following section. A summary of these effects can be found in Figure 2.

3.1. Vitamin E. The potential therapeutic effects of vitamin E in AMI can be comprised of biological actions such as antioxidant and anti-inflammatory effects, as well as a synergism with other antioxidant molecules. Indeed, vitamin E, mainly α-tocopherol, is the major peroxyl radical scavenger in biological lipid-phases such as membranes or LDL [94, 95]. The antioxidant action has been ascribed to its ability to act chemically as a lipid based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation through its own conversion into an oxidized product, α-tocopheroxyl radical. α-Tocopherol can be restored by reduction of the α-tocopheroxyl radical with redox-active reagents like vitamin C or ubiquinol [96]. Otherwise, the tocopheroxyl radical can react with lipids to generate lipid radicals. Therefore, therapeutic uses of α-tocopherol probably require coantioxidants such as vitamin C to have a beneficial effect [97]. The antioxidant effect of vitamin E is not limited to a role of lipid phase ROS scavenger, as it can increase glutathione peroxidase activity [98] and diminish ROS production via downregulating NADPH oxidase [99]. This antioxidant vitamin also has anti-inflammatory effects, by inhibiting the transcriptional activity of NF-κB, a factor able to trigger the expression of proinflammatory genes [100]. The inhibition of the transcriptional activity of NF-κB should also contribute as an anti-inflammatory effect [101]. In addition, α-tocopherol has been shown to inhibit many key events in inflammation such as, but not limited to, platelet aggregation [97] and the release of pro-inflammatory cytokines [102].

It is of interest to mention that vitamin E can exert positive effects reported in clinical studies of revascularization surgeries, such as those of the lower extremities [103], kidney transplantation [104], liver surgery [105], and aortic aneurysm repair [106]. Furthermore, preoperative administration of vitamin E is safe, and this treatment may have beneficial effects by reducing the impact of ischemia-reperfusion injury in liver surgery [107]. Although homologous studies in AMI are still lacking, an amelioration of microvascular impairment in myocardial tissue should be expected, as an effect of pretreatment with vitamin E. However, this effect needs to be tested by further studies.

3.2. Vitamin-C Scavenging Is Concentration Dependent and Requires Intravenous Administration. Plasma vitamin C concentrations do not exceed 100 μmol/L with the ingested amounts found in foods. Even with supplementation approaching maximally tolerated doses, ascorbate plasma concentrations are always <250 μmol/L and frequently <150 μmol/L. By contrast, intravenously injected ascorbate can lead to concentrations of 25–30 mmol/L that are safely achieved [108]. Therefore, in settings accompanied by oxidative stress, such as the myocardial ischemia-reperfusion events, a major beneficial effect of oral administration of vitamin C in the prevention of oxidative damage should not be expected, and intravenous infusion could be considered with this purpose. Indeed, superoxide reacts with NO at a rate 10^5-fold greater than the rate at which superoxide reacts with ascorbic acid [8]. As a consequence, to displace the binding of superoxide anion and NO 10 mmoles/L ascorbate would be needed.

Likely, intracellular levels of ascorbate may not be reflected accurately in its plasma levels since, due to ascorbate transporters, it is accumulated in cells against a concentration gradient by all tissues other than red blood cells [109]. In some tissues, ascorbate levels may exceed the plasma concentrations by as much as 100-fold [110].

3.3. Relationship between Plasmatic Doses and Intracellular Myocardial Concentration: Role of Transporters. Vitamin C is present in the organism in two biological important forms: the reduced form, ascorbic acid, and the oxidized form, dehydroascorbic acid (DHA). Both chemical forms are transported intracellularly [110], a process that requires the participation of specific transporters at the level of the plasma membrane [110]. Once inside the cells, DHA is rapidly reduced to ascorbic acid [111, 112].

Two Na^+-dependent vitamin C transporters (SVCT1 and SVCT2) serve the function of entering ascorbic acid into the cell [113] while DHA transporters are members of the GLUT family of facilitative glucose transporters, with

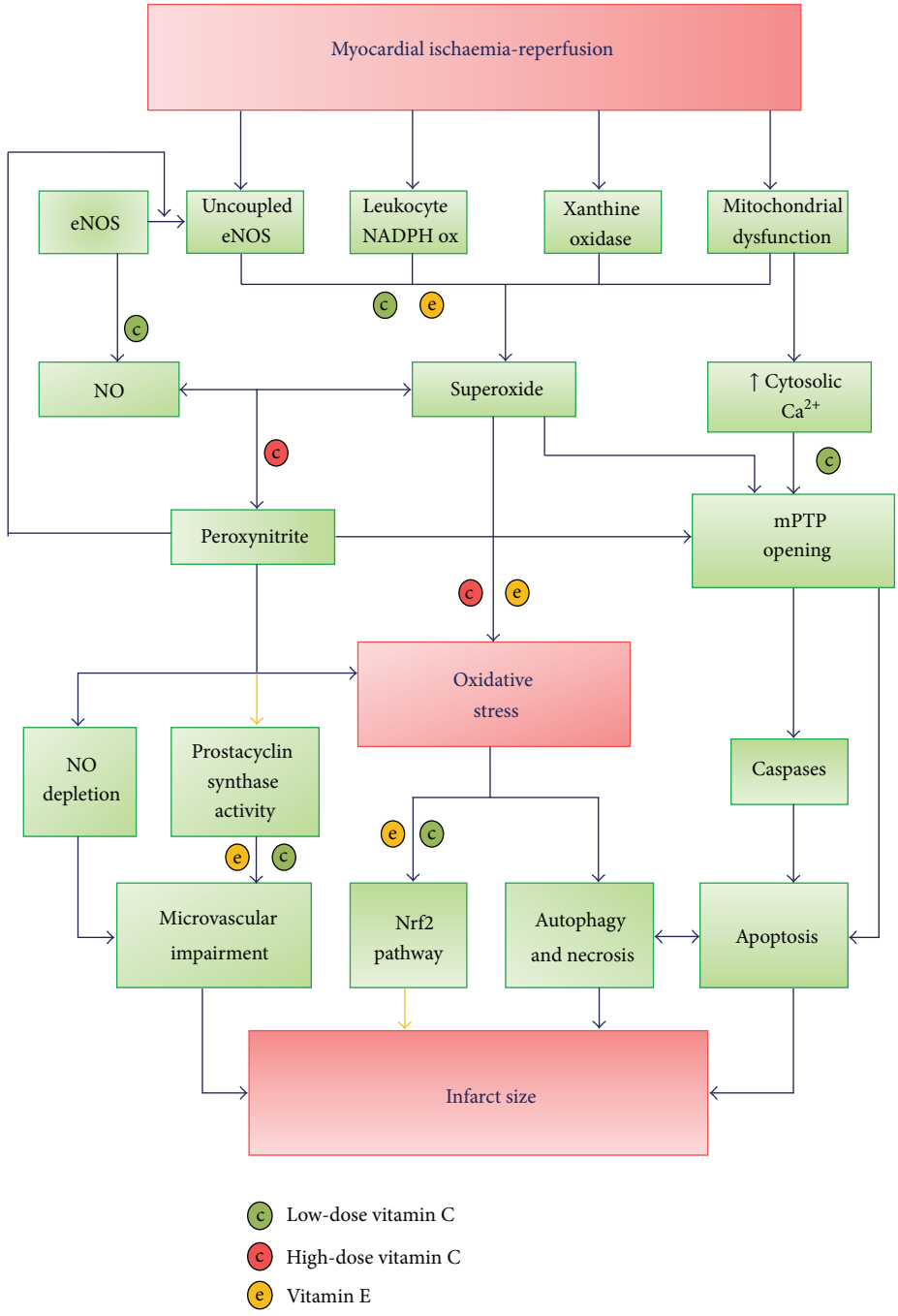

Figure 2: Hypothesis accounting for the acute myocardial infarct size occurring in ischemia-reperfusion through molecular models based on the role of oxidative stress. Abrogation of the deleterious processes by vitamins C and E. Arrow color code: blue stands for "promotion"; yellow for "inhibition": mPTP mitochondrial permeability transition pore; NO; nitric oxide; eNOS; endothelial nitric oxide synthase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADPH ox, oxidized nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor (erythroid-derived 2)-like 2. Adapted from [15], with permissions.

GLUT1, GLUT3, and GLUT4 being DHA transporters [110]. Human myocardium contains GLUT1, GLUT3, and GLUT4 [114].

SVCT1 is confined to epithelial systems including intestine, kidney, and liver, whereas SVCT2 has a widespread location in the body and serves as a host of metabolically active and specialized cells and tissues [113], including the heart [115, 116]. It has been proposed that among the roles of SVCT2 is to provide l-ascorbic acid to protect metabolically active cells from oxidative stress [113].

In accordance with the previously mentioned studies, Guaiquil et al. report an *in vitro* study where adult rat

cardiomyocytes incubated for 30 min with 5 mM DHA accumulated 39 mM ascorbic acid [117].

3.4. Vitamin C and Synergistic Effects with Vitamin E. Vitamin C, ascorbic acid or ascorbate, is a reducing agent that serves as a one-electron donor, generating semidehydroascorbate. When it acts as an antioxidant or enzyme cofactor, it becomes oxidized to DHA [118]. Ascorbate counteracts and prevents the oxidation of lipids, proteins, and DNA, subsequently protecting their structure and biological function. Together with glutathione, ascorbic acid constitutes a primary line of defense against ROS [119].

Ascorbate in aqueous compartments can recycle α-tocopherol in membranes by reducing the α-tocopheroxyl radical back to α-tocopherol [120]. Accordingly, ascorbate has been shown to recycle α-tocopherol in lipid bilayers [121] and erythrocytes [122].

The antioxidant effect of ascorbic acid is not limited to its ability to scavenge ROS. Ascorbate can diminish ROS production through downregulation of NADPH oxidase. The therapeutic potential of vitamin C becomes clear if it considered that the major source of ROS in AMI is their enzymatic production via NADPH oxidase [32, 45, 46]. Vitamin C also suppresses NF-κB activation [123].

In addition, vitamin C prevents the oxidation of tetrahydrobiopterin, a cofactor of NO synthase that is highly sensitive to oxidation. When tetrahydrobiopterin is oxidized, eNOS activity becomes uncoupled, resulting in the production of superoxide instead of NO, thus enhancing the oxidative damage [124].

As it was previously mentioned, concentrations acquired through oral administration of vitamin C doses are not enough to scavenge superoxide anion. Therefore, i.v. administration is required for this purpose.

Impaired microcirculatory reperfusion is improved by vitamin C infusion in hypertension [125] diabetes mellitus [126] and in patients undergoing elective PCA, suggesting that oxidative stress is implicated in such a phenomenon [127]. Also, in patients subjected to thrombolysis following AMI, SOD in the blood was found to be significantly reduced, whereas the activity of the oxidant enzyme, xanthine oxidase, and malondialdehyde levels were found to be significantly increased. However, oral supplementation of vitamin C to the postreperfusion patients restored these parameters back to normal or near normal levels [128].

Even though vitamins C and E exert their individual biochemical effects in water or lipid phases, respectively, they also can interact with each other at the level of interphases, giving rise to synergistic effects of restoring α-tocopherol from α-tocopheroxyl radical [13]. In vitamin-E-supplemented rat hearts, α-tocopherol diminishes rapidly without the addition of vitamin C during reperfusion [129].

4. Clinical Experiences of Antioxidant Treatment of Myocardial Infarction

Experimental studies based on the pathogenic role of ROS and reactive nitrogen species (RNS) in myocardial damage following ischemia-reperfusion events have given promising results for antioxidant cardioprotection. Therefore, it should be expected that treatments with exogenous antioxidant agents could protect the heart against lethal reperfusion injury in clinical models. However, although a number of strategies have been devised to ameliorate this injury, the beneficial effects in the clinical settings have been disappointing up to date [15]. Thus, clinical trials designed to study cardioprotection by long-term administration of vitamins C and E have failed to demonstrate beneficial effects [130–137]. Either thrombolytic therapy or primary percutaneous coronary intervention has proved to be the most effective therapeutic intervention for reducing acute myocardial ischemic injury. However, reperfusion itself can induce cardiomyocyte impairment of structure and function. Consequently, myocardial stunning and even cell death will occur, what is known as myocardial reperfusion injury, for which there is still no effective therapy. Moreover, studies in animal models of AMI suggest that lethal reperfusion accounts for up to 50% of the final myocardial infarct size [7], a damage likely to be preventable.

Randomized, double-blind, placebo-controlled trials with antioxidant therapy using L-carnitine [23] and Coenzyme Q10 [22] as an oral treatment after AMI have suggested a reduction in infarct size and improvement of the clinical outcomes in treated patients (Table 1). Nevertheless these therapies could not primariy prevent lethal reperfusion injury, because of the slow enteric absorption of L-carnitine [138] and Coenzyme Q10 [139]. Some studies have suggested that antioxidant agents attenuate left ventricular remodeling following AMI. Accordingly, in patients with AMI who had undergone primary percutaneous transluminal coronary angioplasty, pretreatment with allopurinol, a xanthine oxidase inhibitor, resulted in effective inhibition of ROS generation and significant improvement of left ventricular ejection fraction at 6 months after PTCA [27]. More recently, administering the ROS scavenger edaravone to patients with AMI immediately prior to reperfusion significantly reduced infarct size and reperfusion arrhythmias [25]. In this study the free radical scavenger was given intravenously and prior to the onset on the reperfusion therapy, thus accounting for the role of oxidative stress in lethal reperfusion injury. However, other attempts, such as intravenous bolus of superoxide dismutase [26], showed no beneficial effect on patients outcome. The authors report that it is possible that a clinically significant benefit might have been missed with such a small sample size because of the heterogeneity of intercoronary collaterals, vascular risk regions, and other important uncontrolled variables. In brief, antioxidants, as well as numerous cardioprotective strategies for reducing lethal reperfusion injury, have failed to provide any benefit to patients during reperfusion heart damage [7].

In regard to the therapeutic use of vitamins C and E with purposes of cardioprotection, although the scientific rationale, epidemiologic data, and retrospective studies have been persuasive, prospective, randomized, placebo-controlled trials have not verified their actual benefit in human diseases [140]. Only one randomized, double-blind, placebo-controlled study has been published using vitamins C and E

Table 1: Cardioprotective strategies using antioxidant vitamins C and E and other antioxidants in acute myocardial infarction.

Details of Study	Study	n	Results	References
		Vitamins C and E		
Vitamin C (1000 mg/12 h infusion) followed by 1200 mg/24 h orally and vitamin E (600 mg/24 h).	Randomized, double-blind, placebo-controlled, multicenter trial.	800	Improvement in mortality and clinical outcomes.	Jaxa-Chamiec et al. (MIVIT trial) [17]
A retrospective analysis of the influence of vitamins C and E on 30-day cardiac mortality in patients with or without DM.	Retrospective study from MIVIT trial.	800 [122 (15%) DM]	Reduction in cardiac mortality in DM patients treated. No significant differences in nondiabetic patients.	Jaxa-Chamiec et al. [18]
Vitamins C and E (600 mg/24 h each) orally on the first day of AMI and lasting for 14 days.	Randomized, double-blind, placebo-controlled trial.	37	Baseline QTd was similar in both groups. Significant decrease in exercise-induced QTd in treated group.	Bednarz et al. [19]
Vitamin A (50,000 IU/24 h), vitamin C (1,000 mg/24 h), vitamin E (400 mg/24 h), and beta-carotene (25 mg/24 h)	Randomized, double-blind, placebo-controlled trial.	125	Reduction in mean infarct size assessed by cardiac enzymes. Improved clinical outcomes.	Singh et al. (the Indian experiment of infarct survival-3) [20]
Vitamins C and E, each 600 mg/24 h orally for 14 days.	Randomized trial.	61	Less ECG alterations in treated patients.	Chamiec et al. [21]
		Other Antioxidants		
Oral treatment with coenzyme Q10 (120 mg/24 h) for 28 days, administered within 3 days of the onset of symptoms.	Randomized, double-blind, placebo-controlled trial.	144	Angina pectoris, total arrhythmias, and poor left ventricular function were significantly reduced.	Singh et al. [22]
Oral L-carnitine (2 g/24 h) for 28 days.	Randomized, double-blind, placebo-controlled trial.	101	Significant reduction in mean infarct size assessed by cardiac enzymes.	Singh et al. [23]
High-dose N-acetylcysteine ($2 \times 1{,}200$ mg/24 h) for 48 h, plus optimal hydration.	Randomized, single-blind, placebo-controlled trial.	251	No differences in any of the end point with N-acetylcysteine or placebo.	Thiele et al. [24]
30 mg edaravone intravenously before reperfusion.	Randomized, placebo-controlled trial.	101	Significant reduction in reperfusion arrhythmia and mean infarct size assessed by cardiac enzymes.	Tsujita et al. [25]
Intravenous bolus of superoxide-dismutase (10 mg/kg of body weight) followed by a 60 min infusion of 0.2 mg/kg/min before PCI.	Randomized, placebo-controlled trial.	120	No significant differences.	Flaherty et al. [26]
Allopurinol (400 mg) administered orally just after the admission (approximately 60 min before reperfusion).	Randomized trial.	38	Slow flow in the recanalized coronary artery after PTCA occurred less frequently.	Guan et al. [27]

*AMI: acute myocardial infarction; DM: diabetes mellitus; PCI: percutaneous coronary intervention; QTd: QT dispersion in electrocardiogram; ECG: electrocardiogram; PTCA: percutaneous transluminal coronary angioplasty.

before the reperfusion therapy [17]. They used 1000 mg/12 hr infusion of vitamin C followed by 1200 mg/24 hr orally and vitamin E (600 mg/24 hr) for 30 days. Results suggest that supplementation with these antioxidants seems to positively influence the clinical outcome of patients with AMI, in terms of composite of in-hospital cardiac mortality, nonfatal new myocardial infarction, ventricular tachycardia, ventricular fibrillation, asystole or shock, and pulmonary edema. Furthermore in a retrospective analysis of the aforementioned data [18] a significant reduction in 30-day cardiac mortality in diabetic patients treated with vitamins C and E has been found. In patients without diabetes, the administration of vitamins had no such effect on cardiac mortality. This result on diabetic patients with AMI seems to be particularly reasonable because of the increased ROS formation known to occur in these patients [141]. It should be noted that the authors acknowledged that the dose of vitamin C used only raised plasma levels to 0.1 mmol/L. Thus, the most important function to abrogate oxidative stress-dependent processes cannot be achieved by vitamin C doses used in this study. Indeed, it is necessary to reach plasma levels of ascorbate about 10 mmol/L to prevent chemical reaction of NO and superoxide anion, otherwise resulting in a highly peroxidant pathway [8].

It is of interest to remark that up to date the available clinical trials have been designed with significant methodological deficiencies that demand cautious interpretation of these results. Nevertheless, some beneficial effects derived from the biological properties of antioxidant vitamins could be expected in the patients subjected to these protocols, depending on the dose and administration manner. Taking into consideration the high reactivity of ROS, their short life span, their continuous production in close proximity to biological targets, and their ability to be modified into other more reactive species, one realizes that, in order to cope with these deleterious metabolites, the antioxidant therapy should be administered to the body continuously, in high concentrations, and targeted to the biological site susceptible to oxidative damage. In addition, to scavenge ROS efficiently, antioxidants must be present at the location of radical formation in order to compete with the biological target [51]. Therefore, antioxidant therapy should be designed carefully [142–145]. On the other hand, an understanding of the mechanism of the activity of scavengers, including their mutual collaboration, synergistic activity, and interrelationships, prompts the suggestion that the antioxidant be given in combinations, such as preparations of multiscavenger in both oxidized and reduced forms and with no transition metals in the formulations. They should be designed in appropriate pharmaceutical dosage forms such as sustained-release formulations. One has to be aware of their potential side effects and their upper toxic dose, which can easily be reached, because these compounds are widely distributed in our diet [51].

5. Conclusions and Perspectives

Cumulated data strongly suggest the major contribution of ROS to the development of oxidative damage in myocardial infarction followed by revascularization, a clinical model of oxidative stress. Nevertheless, the clinical trials aimed to test the therapeutic role of antioxidants in reperfusion damage have failed to find a beneficial effect. These disappointing results for the case of vitamins C and E could be explained on the basis of the methodological design of the protocols. Oral doses of vitamin C are not suitable to reach plasma levels high enough to scavenge ROS (i.e., above 10 mmoles/L). Although these levels could be achievable by massive infusion of ascorbate, up to date no protocols have been performed with this purpose. It is of particular relevance to consider that (i) vitamin E, mainly α-tocopherol, is the major peroxyl radical scavenger in biological lipid phases such as membranes or LDL; (ii) α-tocopherol can be restored by reduction of the α-tocopheroxyl radical with redox-active reagents like vitamin C; (iii) infusion of ascorbate at a rate high enough to scavenge ROS could offer an unexplored therapeutic opportunity in short-term surgical procedures involving major risk of oxidative stress and its consequences; (iv) although a scavenging effect at oral doses of vitamin C should not be expected, decreased ROS production could result from its ability to downregulate NADPH oxidase activity, an effect shared by vitamin E; (v) stabilization of tetrahydrobiopterin, a cofactor of eNOS, could also be achieved by oral doses of vitamin C; otherwise the enzyme could produce superoxide instead of NO; and (vi) the whole molecular effects of vitamins C and E could account for an abrogation of the microvascular adverse events occurring in the percutaneous coronary angioplasty, as well as in other surgical procedures involving ischemia-reperfusion events. It is noticeable that these safe, easily available, low cost naturally occurring substances could improve the clinical outcome of patients subjected to percutaneous angioplasty, a novel view likely to give rise to the performance of clinical trials devised to demonstrate the validity of this paradigm.

Abbreviations

AMI: Acute myocardial infarction
AMPK: 5′Adenosine monophosphate-activated protein kinase
CAT: Catalase
CRP: C-reactive protein
DHA: Dehydroascorbic acid
GSH: Reduced glutathione
GSH-Px: Glutathione peroxidase
H_2O_2: Hydrogen peroxide
IL-6: Interleukin-6
IL-1β: Interleukin-1β
MAPK: Mitogen-activated protein kinase
MCP-1: Chemokine monocyte chemoattractant protein-1
MCPIP: MCP-1-induced protein
mPTP: Mitochondrial permeability transition pore
MPO: Myeloperoxidase
mTOR: Mammalian target of rapamycin
NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells
NO: Nitric oxide

OH·: Hydroxyl radical
$ONOO^-$: Peroxynitrite
PCA: Percutaneous coronary angioplasty
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TNF-α: Tumour necrosis factor-α.

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Research Article

Antioxidant and α-Amylase Inhibitory Property of *Phyllanthus virgatus* L.: An *In Vitro* and Molecular Interaction Study

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The present study on *Phyllanthus virgatus*, known traditionally for its remedial potential, for the first time provides descriptions of the antioxidant and inhibition of α-amylase enzyme activity first by *in vitro* analyses, followed by a confirmatory *in silico* study to create a stronger biochemical rationale. Our results illustrated that *P. virgatus* methanol extract exhibited strong antioxidant and oxidative DNA damage protective activity than other extracts, which was well correlated with its total phenolic content. In addition, *P. virgatus* methanol extract strongly inhibited the α-amylase activity (IC_{50} 33.20 ± 0.556 μg/mL), in a noncompetitive manner, than acarbose (IC_{50} 76.88 ± 0.277 μg/mL), which showed competitive inhibition. Moreover, this extract stimulated the glucose uptake activity in 3T3-L1 cells and also showed a good correlation between antioxidant and α-amylase activities. The molecular docking studies of the major bioactive compounds (9,12-octadecadienoic acid, asarone, 11-octadecenoic acid, and acrylic acid) revealed via GC-MS analysis from this extract mechanistically suggested that the inhibitory property may be due to the synergistic effect of these bioactive compounds. These results provide substantial basis for the future use of *P. virgatus* methanol extract and its bioactive compound in *in vivo* system for the treatment and management of diabetes as well as in the related condition of oxidative stress.

1. Introduction

Oxidative stress induced by reactive oxygen species (ROS) can cause cell membrane disintegration, protein, lipid, and deoxyribose nucleic acid (DNA) damage which can further initiate or propagate the development of many chronic and degenerative diseases [1–3]. When there is imbalance between ROS generation and antioxidant protection mechanism, it leads to cellular dysfunction causing various diseases inducing diabetes mellitus (DM) [4, 5]. Diabetes is an important metabolic syndrome affecting about 200 million people worldwide. The critical effect of diabetes is postprandial hyperglycemia and reduction in antioxidant defense mechanism. So, the management of type 2 DM could be done both by reducing oxidative stress as well as by delaying the absorption of glucose through the inhibition of any one of the carbohydrates-hydrolyzing enzymes, α-glucosidase, and α-amylase that are responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption [6–8].

There has been enormous interest in natural antioxidants due to their ability to neutralize the effects of ROS that are not only responsible for alleviating the oxidative stress condition in diabetes but are also helpful in managing the postprandial hyperglycemia. The growing interest to combat the side effect of the drugs available for diabetes leads to the development of green medicines due to their higher stability, higher antioxidant potential, low cost, and low cytotoxicity. Plants are rich sources of phytochemicals, which possess a variety of biological activities including antioxidant and antidiabetic potential both *in vitro* and *in vivo* [7–12].

In the last few decades, plants of genus *Phyllanthus* (Euphorbiaceae) came in focus due to their wide distribution, diversity in the genus, broad therapeutic potential, and

variety in the secondary metabolites [13]. This family includes several plant species among all the species; *P. amarus*, *P. urinaria*, *P. maderaspatensis, P. virgatus,* and *P. fraternus* are the most popular ones due to their antioxidant properties as well as their extensive use in the treatment of disease related to kidney, liver, urinary bladder, intestinal infection, cancer, and diabetes [13–18].

It has been previously reported that *P. virgatus* is rich in polyphenols [13] and is known traditionally for its antioxidant [14], antimicrobial, antiseptic, anti-inflammatory agent [19], and anticancer activity [20]. The antidiabetic properties of various *Phyllanthus* species have been investigated in experimental models [15, 21]. However, only one study speculated the antidiabetic property of *P. virgatus* [21], and still the detailed investigation pertaining to their mechanism of action is lacking. So, this study was the first integrative approach to investigate and correlate the antioxidant, oxidative DNA damage protective activity, α-amylase inhibitory and glucose uptake property of various extracts of *P. virgatus*. Moreover, the mechanistic aspect of these compounds, elucidated via GC-MS analysis, was explored by carrying out docking studies against porcine pancreatic α-amylase in an array to give molecular mechanism of action of such inhibitors.

2. Methods

2.1. Chemicals. Chemicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), ascorbic acid, thiobarbituric acid (TBA), Folin-Ciocalteu reagent (FCR), dimethyl sulfoxide (DMSO), dinitro salicylic acid (DNS), Dulbecco's modified Eagle medium (DMEM), dexamethasone (DEX), isobutylmethylxanthine (IBMX), foetal bovine serum (FBS), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from HiMedia Laboratories, Mumbai, India. Porcine pancreatic α-amylase was procured from SRL Pvt. Ltd., Mumbai, India. Methanol (MeOH), acetone, dichloromethane (DCM), *n*-hexane (*n*-hex), ethyl acetate (EtOAc), pUC18 plasmid, and Merckotest GOD/POD kit were obtained from Merck, India. Acarbose was obtained from Bayer Pharmaceuticals, and actrapid insulin was purchased from Torrent Pharmaceuticals Ltd., India. All chemicals were of analytical grade.

2.2. Collection and Preparation of Plant Extract. *P. virgatus* whole plant was collected from the local area around Integral University, Lucknow, India, in the months of July-August. The plant was botanically identified and authenticated by Dr. Mohd. Tariq, National Botanical Research Institute, Lucknow, India, and a voucher specimen (98195) of the plant was submitted there. *P. virgatus* whole plants were shed dried and made in coarse powder, avoiding sun dried due to the signature modification of the biochemicals. The dried powder (25 g) of the plants was extracted using nonpolar, partially polar, and polar solvents successively with the required amount of each of *n*-hex, DCM, EtOAc, MeOH, and water solvents in soxhlet apparatus until it turned colorless. The solvent was removed, filtered, and dried at room temperature, and residues were scratched out and stored at −20°C for future use. The percentage yield of different fractions was calculated by using the formula

$$\%\ \text{yield} = \frac{\text{weight of crude extract}}{\text{weight of raw material}} \times 100. \quad (1)$$

The percentage yield was found to be *n*-hex: 2.17%, DCM: 0.75%, EtOAc: 0.37%, MeOH: 6.71%, and water: 2.31%.

2.3. Phytochemical Screening and Estimation of Total Phenol Content. Qualitative chemical tests were carried out to identify the phytochemicals present in various extracts of *P. virgatus* using standard procedure [22]. Total phenol content (TPC) of the extracts was determined by using Folin-Ciocalteu method [23].

2.4. DPPH Radical Scavenging Activity. The DPPH radical scavenging capacity of the various extracts of *P. virgatus* was determined by the method of Brand-Williams et al. [24]. Ascorbic acid was used as a reference standard. Percent (%) scavenging of DPPH free radical was measured using the following equation:

$$\%\ \text{DPPH radical scavenging} = \left[\frac{(\text{absorbance of control} - \text{absorbance of test sample})}{(\text{absorbance of control})}\right] \times 100. \quad (2)$$

Further, IC_{50} value represented the concentration of the extract that caused 50% inhibition of DPPH radicals and was calculated by interpolation of linear regression analysis.

2.5. Ferric Reducing Antioxidant Potential. A modified method of Benzie and Strain [25] was adopted to determine the ferric reducing antioxidant potential (FRAP) of various extracts of *P. virgatus*. Briefly, the FRAP reagent was freshly prepared by mixing sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution (in 40 mM HCl), and 20 mM Fe(III) chloride solution in a volume ratio of 10 : 1 : 1, respectively. Hundred microliters of the sample at various concentrations was added to 3 mL of the FRAP reagent. The absorbance was measured after 30 min at room temperature at 593 nm. The standard curve was plotted using $FeSO_4$ solution, and results were expressed as μmole Fe(II)/g dry weight of plant material.

2.6. Hydroxyl Radical Scavenging Assay. Hydroxyl radical scavenging activity of various extracts of *P. virgatus* was evaluated by the method of Badami et al. [26]. The percentage of hydroxyl radical scavenging potential was calculated by

using the following formula, and IC_{50} was calculated as described previously:

$$\% \text{ Hydroxyl radical scavenging activity} = \left[\frac{(\text{absorbance of control} - \text{absorbance of test sample})}{(\text{absorbance of control})}\right] \times 100. \quad (3)$$

2.7. DNA Protection Assay. DNA protection assay was performed using supercoiled pUC18 (2686 bp) plasmid DNA according to the method of Lee et al. [27] with slight modifications. Plasmid DNA (250 ng) was incubated with Fenton's reagent containing H_2O_2 (30 mM), ascorbic acid (100 μM), and $FeCl_3$ (160 μM) in the presence and absence of the plant extract, and the final volume of the mixture was raised up to 20 μL. The mixture was then incubated for 45 min at 37°C followed by the addition of loading dye, and the electrophoresis was carried out in Tris-acetate-EDTA buffer (40 mM Tris base, 16 mM acetic acid, and 1 mM EDTA; pH 8.0). DNA was analyzed followed by ethidium bromide staining, and mannitol was used as positive control.

2.8. α-Amylase Inhibition Assay. To determine the *in vitro* α-amylase inhibition by various extracts of *P. virgatus,* the standard procedure [28] was adopted with slight modification. Briefly, porcine pancreatic α-amylase was dissolved in ice-cold phosphate buffer (20 mM), pH 6.7, containing sodium chloride (6.7 mM) to give a concentration of 0.15 unit/mL. Triplicate test tubes including the blank were prepared. In each test tube, 250 μL of the enzyme preparation was mixed with 100 μL of each of the extracts except the blank. The mixtures were stirred in a vortex and preincubated in a water bath at 37°C for 20 minutes. After incubation, 250 μL of the substrate preparation (0.5% w/v starch in 20 mM phosphate buffer; pH 6.7) was transferred into each test tube to start the reaction. The mixture was vortexed and then incubated at 37°C for 15 minutes. Two mL of DNS color reagent (DNS 40 mM, K-Na tartrate 1 M, and sodium hydroxide 0.4 M) was added, vortexed and boiled in a water bath at 100°C for 10 minutes. Thereafter, the mixture was cooled down, and the absorbance was read at 540 nm. Acarbose was used as standard inhibitor.

Inhibition rates were calculated as percentage controls using the formula:

$$\% \text{ inhibition} = 100 - \% \text{ reaction}, \quad (4)$$

where % reaction = (mean product in sample/mean product in control) × 100.

Further, IC_{50} value represented the concentration of the extract that caused 50% inhibition of α-amylase and was calculated by interpolation of linear regression analysis.

2.9. Determination of Mode of Inhibition. Mode of inhibition of *P. virgatus* methanol extract against α-amylase was determined by the method of Mogale et al. [29]. For the assay, two sets (A and B) of 6 duplicate test tubes were prepared to determine the enzyme activity in the presence [set A] and absence [set B] of an inhibitor (methanol extract/standard acarbose). In set A, 100 μL of the inhibitor (plant extract or acarbose, 1 mg/mL) solution was added in each test tube except the blank; this was followed by the addition of 100 μL of the enzyme porcine α-amylase (0.15 units/mL). In set B, 100 μL of phosphate buffer (20 mM), pH 6.7, containing sodium chloride (6.7 mM) was added in each test tube followed by 100 μL of the enzyme solution. Both sets of test tubes were thoroughly mixed in a vortex mixer and preincubated in a water bath at 37°C for 20 minutes. Serial dilutions of the substrate solution were added in both sets of test tubes with concentration ranging between 2.5 μg/mL and 0.156 μg/mL. All the tubes were then incubated at 37°C for 15 minutes, followed by the addition of 2 mL of DNS color reagent and the mixtures were boiled for 10 minutes. Absorbance of the colored solution was read at 540 nm. Double reciprocal curve ($1/V$ *v/s* $1/[S]$) for both sets was plotted to determine the effect of the plant extract/acarbose on V_{max} and K_m of the enzyme, where V and $[S]$ are, respectively, the velocity of the reaction and substrate concentration.

2.10. Cell Culture. 3T3-L1 preadipocytes cell lines are known to mimic *in vivo* organs that have an influence on glucose homeostasis. These cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were routinely cultured at 37°C in a humidified 5% CO_2, 95% air atmosphere and were grown in DMEM medium supplemented with 10% FBS, L-glutamine (8 mM), and 2% antimycotic.

2.11. Cell Viability Assay. The standard MTT colorimetric assay [30], which is based on the reduction of MTT by mitochondrial dehydrogenase to a purple formazan product, was used to assess the cytotoxic activity of *P. virgatus* methanol extract. The effect was quantified as follows:

$$\% \text{ inhibition} = \frac{\text{absorbance of the control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100. \quad (5)$$

2.12. Glucose Uptake Assay. Cells were cultured and plated at a density of 12,000 cells/well in a 24-well plate and incubated for 24 hours in the DMEM growth media containing 5 mM glucose. On day 1, the growth medium was replaced by supplemented medium, which consisted of DMEM supplemented with 10% FCS, insulin (10 μg/mL), DEX (10^{-8} M), and IBMX (0.1 mM). Cells were refed 48 hours later with the same supplemented medium, and after another 24 hours (day 4), this medium was removed and replaced with growth medium including the treatment protocol [11] (Table 1). After a further 48-hour incubation (day 6), the cells were assayed in their appropriate experiments. Ten microliters of the media was removed and placed in the 96-well plates to which 200 μL of GOD/POD reagent was added and incubated for 15 min

at 37°C. The change in the color was recorded at 495 nm. The following equation was used to calculate the glucose content (mg/dL) in each well. Concentration of unknown sample = (concentration of sample/abs. of standard − abs. of reagent blank) × abs. of unknown sample − abs. of reagent blank). Finally, glucose uptake over control was calculated as the difference between the initial and final glucose content in the incubated medium.

2.13. Gas Chromatography and Mass Spectroscopy (GC-MS) Analysis. In order to know the bioactive metabolites responsible for antioxidant and antidiabetic activity, the methanol extract of *P. virgatus* was subjected to GC-MS analysis. The sample was injected into an RTX-5 column (60 m × 0.25 mm i.d., film thickness 0.25 μm) of GC-MS (model GC-MS-QP-2010 plus, Shimadzu Make). Helium was used as carrier gas at a constant column flow of 1.2 mL/min at 173 kPa inlet pressure. Temperature programming was maintained from 100°C to 200°C with constant rise of 5°C/min and then held isothermal at 200°C for 6 min; further, the temperature was increased by 10°C/min up to 290°C and again held isothermal at 290°C for 10 min. The injector and ion source temperatures were 270°C and 250°C, respectively. Mass spectra were taken at 70 eV a scan interval of 0.5 s and fragments from 40 to 950 Dalton. The final confirmation of constituents was made by computer matching of the mass spectra of peaks with the National Institute of Standards and Technology (NIST) libraries mass spectral database.

2.14. Docking Analyses

2.14.1. Preparation of Enzyme and Ligand. The crystal structure of porcine pancreatic α-amylase (PDB ID: 1DHK) in complex with acarbose was retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) protein databank. Water molecules as well as other heteroatoms were removed, and the protein was subjected to energy minimization using CharMM force field [31]. The method deployed for energy minimization was steepest descent at RMS gradient of 0.1 for 1000 steps. Compounds, whose 3-D structures were available, were extracted from PubChem compounds database, while those whose structures were not present were drawn using ChemDraw like hexadecanoic acid, 9,12-octadecadienoic acid, 11-octadecenoic acid, and 6-octadecynoic acid.

2.14.2. Molecular Docking. Molecular docking studies were carried out using AutoDock program [32] to get the favorable binding modes for compounds within the active site of porcine pancreatic α-amylase. Before conducting molecular docking, validation was performed, in which the acarbose present within the binding site of porcine α-amylase in crystal structure was extracted. This acarbose was subjected to redocking within the active site of porcine α-amylase using AutoDock. The binding confirmation was visualized using PyMOL. After complete execution of AutoDock, various conformations of ligand in complex with the receptor were obtained, which were finally ranked on the basis of binding energy.

Table 1: Treatment protocol for glucose uptake assay.

S. no.	Incubation medium
Group 1	1000 μL DMEM containing 5 mM glucose
Group 2	900 μL DMEM + 100 μL insulin (1 IU/mL)
Group 3	900 μL DMEM + 100 μL metformin (1 mg/1 mL)
Group 4	900 μL DMEM + 100 μL plant extract (1 mg/mL)
Group 5	800 μL DMEM + 100 μL insulin (1 IU/mL) + 100 μL plant extract (1 mg/mL)
Group 6	800 μL DMEM + 100 μL insulin (1 IU/mL) + 100 μL metformin (1 mg/1 mL)
Group 7	700 μL DMEM + 100 μL insulin (1 IU/mL) + 100 μL metformin (1 mg/1 mL) + 100 μL plant extract (1 mg/mL)

2.15. Statistical Analysis. The results were analyzed by using one-way analysis of variance (ANOVA) and two-tailed Student's t-test. Statistical significance was expressed as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P > 0.001$.

3. Results

3.1. Phytochemical Estimation and Total Phenol Content. Our results illustrated significant presence of tannins, terpenoids, saponins, phenols, carbohydrate, flavanoids, protein, glucose, and reducing sugar in *P. virgatus* methanol extract (Table 2). Water extract contains all the above phytochemicals except glucose and reducing sugar. In addition, EtOAc extract contains terpenoids, flavanoid, protein, glucose, and reducing sugar, while only tannins, terpenoids, and protein were present in DCM extracts. In contrast, *n*-hex contains only protein content. The TPC (μg/mg GAE) in the various extracts of *P. virgatus* was also determined and found to be in the following decreasing order: MeOH > water > EtOAc > *n*-hex > DCM. From the data, it is evident that methanol extract has higher phenolic content (176.68 ± 0.032 μg GA/mg plant extract) than the water and ethyl acetate extracts, whereas DCM and *n*-hex have the lowest phenolic content (Table 3).

3.2. Total Antioxidant Activity. Antioxidant activities of different *P. virgatus* extracts were assessed by FRAP assay, which is based on their ability to reduce ferric ions to ferrous form. The results illustrated that methanol extract has significantly higher FRAP values (28.61 ± 0.2184 μmol Fe(II)/g) as compared to other extracts (0.4–6 μmol Fe(II)/g) and standard ascorbic acid (Table 3). A simple linear regression analysis

Table 2: Phytochemical constituents of sequentially extracted *P. virgatus* fractions.

P. virgatus extracts	Tannins	Terpenoids	Phenols	Flavanoids	Protein	Glucose	Reducing sugar
n-hex	– –	– –	– –	– –	++	– –	– –
DCM	++	+	– –	– –	++	– –	– –
EtOAc	– –	++	– –	+++	++	+	+
Water	+++	+	++	+++	+++	– –	–
MeOH	++	++	++	+++	+	++	+

Table 3: Ferric reducing antioxidant potential and total phenol content of *P. virgatus* extracts. The data represents mean ± S.D. of six FRAP and three TPC experiments.

Extracts/reference	FRAP value (μmol Fe(II)/g)	TPC (μg GA/mg extract)
n-hex	0.442 ± 0.126	2.61 ± 0.017
DCM	0.924 ± 0.419	1.92 ± 0.001
EtOAc	1.255 ± 0.53	37.42 ± 0.010
MeOH	28.61 ± 0.2.184	176.68 ± 0.032
Water	5.69 ± 9.7	67.766 ± 0.029
Ascorbic acid	13.05 ± 3.131	

Table 4: IC_{50} values of *P. virgatus* against DPPH radicals, hydroxyl radicals, and α-amylase activity.

Activity	Plant extract/standard	IC_{50} (μg/mL)
DPPH radical scavenging	*P. virgatus* (MeOH)	18.59 ± 0.515
	P. virgatus (water)	40.36 ± 2.35
	P. virgatus (EtOAc)	NS
	P. virgatus (DCM)	NS
	P. virgatus (*n*-hex)	NS
	Ascorbic acid	10.72 ± 0.33
Hydroxyl radical scavenging	*P. virgatus* (MeOH)	12.53 ± 2.38
	P. virgatus (water)	14.56 ± 0.389
	Mannitol	NS
α-Amylase inhibition	*P. virgatus* (MeOH)	33.20 ± 0.556
	Acarbose	76.88 ± 0.277

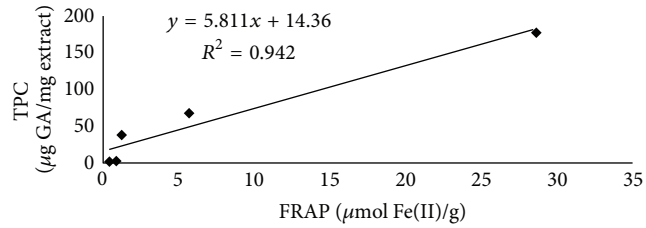

Figure 1: Linear correlation between the amount of TPC and antioxidant capacity (FRAP) of *P. virgatus* in various solvent systems.

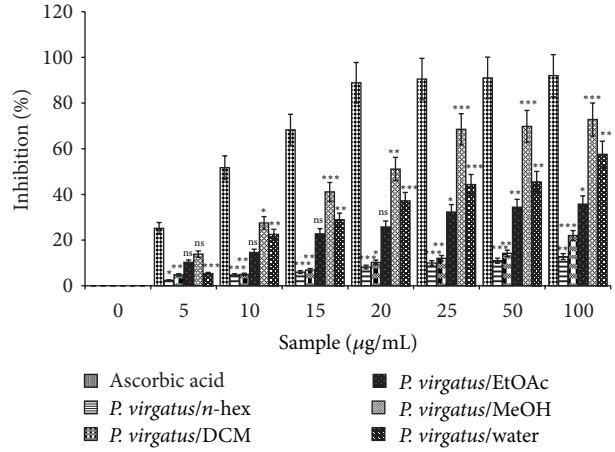

Figure 2: DPPH radical scavenging activity of different extracts of *P. virgatus* and standard ascorbic acid. The data represent percent scavenging of DPPH radicals. The results are mean ± S.D. of three parallel measurements. Nonsignificant (ns), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 μg/mL.

was used to analyze the correlation between FRAP value and the TPC. Figure 1 showed linear corelation between the total phenols and the FRAP value. The coefficient of determination was 0.942 between the TPC and the antioxidant (FRAP) value, indicating that the antioxidant capacity of the extracts can be attributed to their phenolic compounds.

3.3. DPPH Radical Scavenging Activity. The relatively stable DPPH radical is widely used to evaluate the free radical scavenging activity of various natural antioxidants including plant extracts. The data present in Figure 2 showed the percent inhibition of DPPH radical scavenging activity of different extracts of *P. virgatus*. The methanol fraction of *P. virgatus* exhibited higher antioxidant activity with an IC_{50} value of 18.59 ± 0.515 μg/mL as compared to ascorbic acid (Table 4). IC_{50} value of EtOAc, DCM, and *n*-hex extract failed to show any significant scavenging activity, whereas the water extract showed an IC_{50} value of 40.36 ± 2.35 μg/mL. From the data, we observed that DPPH radical scavenging activity was increased as the concentration increased for each individual extract, with marked increase in methanol extract (Figure 2).

3.4. Hydroxyl Radical Scavenging and Oxidative DNA Damage Protective Activity. Hydroxyl radicals ($OH^{\bullet}$) are singlet oxygen species, which are highly reactive and cause damage to various biological macromolecules. Therefore, the role of different extracts of *P. virgatus* in directly scavenging and in protecting the DNA damage, caused by hydroxyl radical, was evaluated. The data presented in Figure 3 clearly indicates better scavenging activity of the methanol extract with an IC_{50} value of 12.53 ± 2.38 μg/mL than water extract,

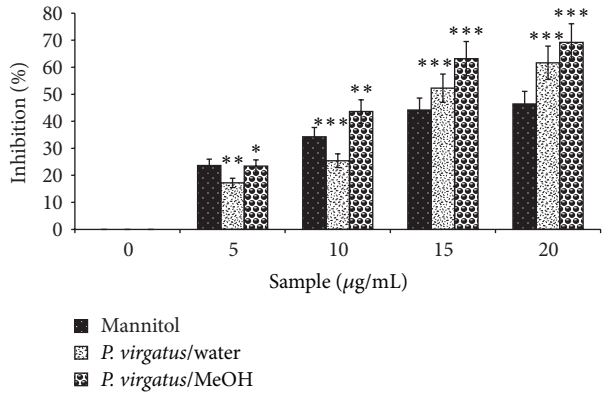

Figure 3: Hydroxyl radical scavenging activity of the *P. virgatus* MeOH, water extract, and reference compound mannitol. The data represents the percentage of inhibition of deoxyribose degradation. The results are expressed as mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 μg/mL.

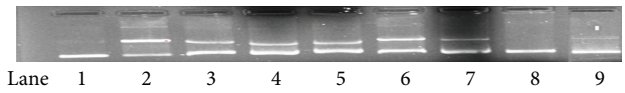

Figure 4: Effect of *P. virgatus* water and MeOH extracts on damaged supercoiled pUC18 plasmid DNA. Lane 1: pUC18 DNA + PBS; lane 2: pUC18 DNA + Fenton's reagent; lane 3: DNA + Fenton's reagent + water extract (50 μg/mL); lane 4: DNA + Fenton's reagent + water extract (100 μg/mL); lane 5: DNA + Fenton's reagent + water extract (200 μg/mL); lane 6: DNA + Fenton's reagent + MeOH extract (50 μg/mL); lane 7: DNA + Fenton's reagent + MeOH extract (100 μg/mL); lane 8: DNA + Fenton's reagent + MeOH extract (200 μg/mL); lane 9: Mannitol (200 μg/mL).

while the other extracts (data not shown) including mannitol showed insignificant scavenging activity of hydroxyl radical.

The oxidative DNA damage protective activity of *P. virgatus* methanol and water extracts showed almost complete and partial protection of $OH^{\bullet}$-induced oxidatively damaged plasmid DNA, respectively (Figure 4). Incubation of pUC18 plasmid DNA with Fenton's reagent resulted in the cleavage of supercoiled form to give open circular and linear forms of plasmid DNA, indicating that OH generated from iron-mediated decomposition of H_2O_2 produced both single-strand and double-strand DNA breaks. Addition of *P. virgatus* methanol and water extracts (50,100 and 200 μg/mL) showed complete and partial protection of supercoiled DNA (Figure 4).

3.5. α-Amylase Inhibitory Property. In an array to explore the antidiabetic activity, various extracts of *P. virgatus* were screened for the α-amylase inhibitory property. Initial screening of various extracts showed that the methanol extract has significantly higher percentage of α-amylase inhibition, that is, 43.2% and 66.09% at 25 and 50 μg/mL, respectively (Figure 5). Furthermore, the methanol extract showed concentration-dependent increase in percent inhibition of α-amylase activity and also exhibited a lower IC_{50} value

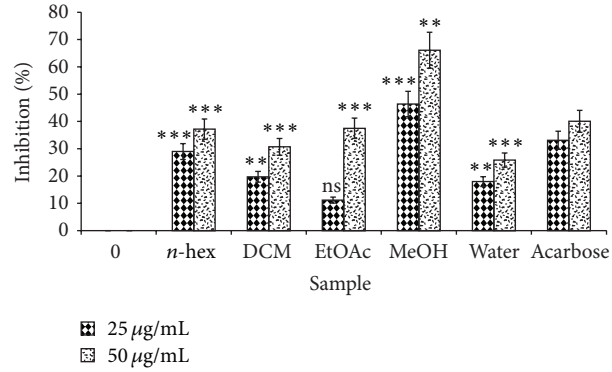

Figure 5: Screening of α-amylase inhibitory property of various extracts of *P. virgatus*. Results are mean ± S.D. of three parallel measurements. Nonsignificant (ns), $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 μg/mL.

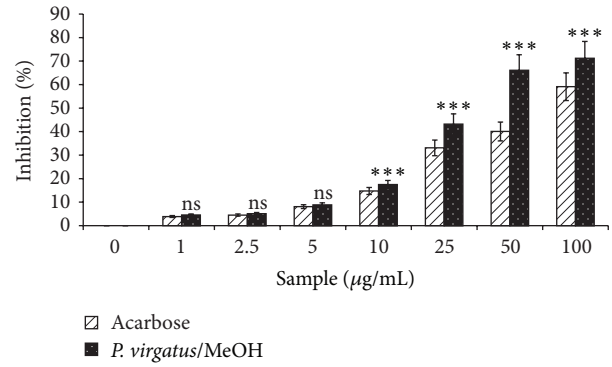

Figure 6: Concentration-dependent inhibition of *P. virgatus* methanol extract and reference compound acarbose. The results are expressed as mean ± S.D. of three parallel experiments. Nonsignificant (ns), $^{***}P < 0.001$ versus 0 μg/mL.

(33.20 ± 0.556 μg/mL) than standard drug acarbose, which in turn indicates a potent antidiabetic property of this extract (Figure 6; Table 4).

The mode of inhibition of acarbose and methanol extracts of *P. virgatus* against porcine α-amylase was also determined by means of Lineweaver-Burk double reciprocal plot of $1/v$ versus $1/[S]$. The methanol extract showed a noncovalent type of noncompetitive inhibition against porcine α-amylase (Figure 7(b)), whereas acarbose was competitive in nature (Figure 7(a)). In addition, a linear correlation was also observed between DPPH radical scavenging activity and α-amylase inhibitory property of *P. virgatus* methanol extract ($R^2 = 0.885$) (Figure 8).

3.6. Glucose Uptake Assay. It was found that the methanol extract of *P. virgatus* showed strong α-amylase inhibitory property; further, its role in glucose utilization in differentiated 3T3-L1 adipocytes cell line was also studied *in vitro*. The ability of the plant extract to induce glucose uptake was tested in different combinations (Table 1), and it was found that the methanol extract of *P. virgatus* alone showed significant

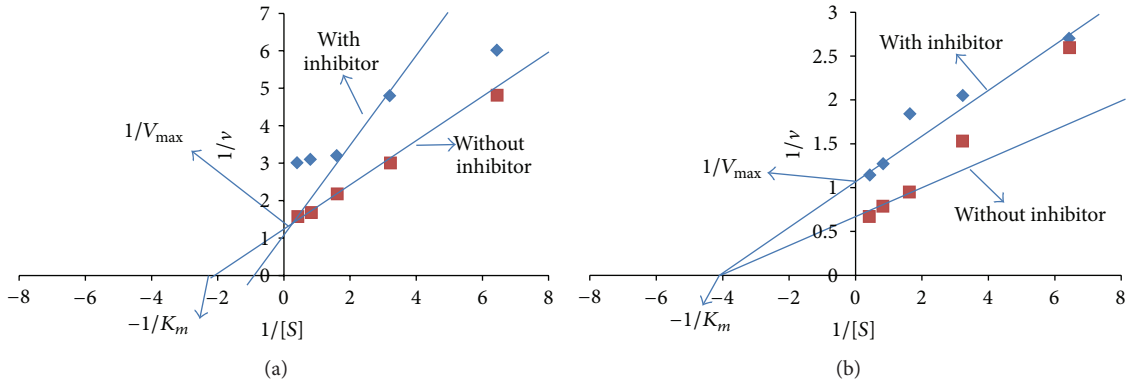

FIGURE 7: Lineweaver-Burk double reciprocal plot of $1/v$ versus $1/[S]$ of acarbose (a) and *P. virgatus* methanol extract (b) against α-amylase.

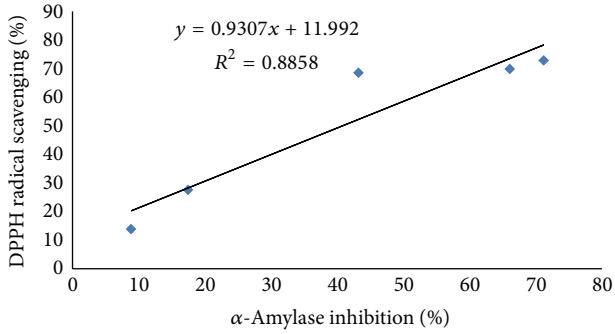

FIGURE 8: Linear correlation between the DPPH radical scavenging activity and α-amylase inhibition of *P. virgatus* methanol extract.

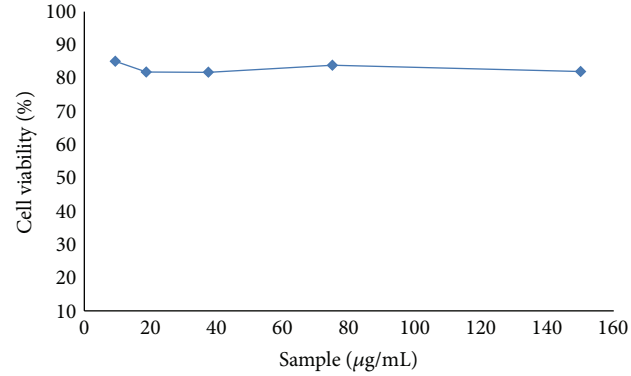

FIGURE 10: Percent of cell viability of 3T3-L1 adipocytes treated with methanol extract of *P. virgatus* at various concentrations.

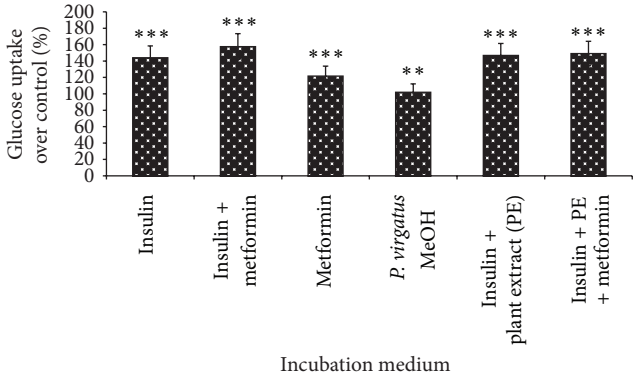

FIGURE 9: Effect of *P. virgatus* methanol extract on glucose utilization in differentiated 3T3-L1 cell line. Results are mean ± S.D. of three parallel measurements. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significance compared to unstimulated cells.

glucose utilization up to 102%; in the presence of insulin it increases up to 146.8%, and in the presence of both insulin and metformin, the glucose uptake of extract was markedly increased by 149% over the control value. In addition, insulin and metformin alone showed 144% and 122% of glucose uptake, while 158% of glucose uptake was observed during their synergistic treatment (Figure 9).

Moreover, to verify the cytotoxicity of methanol extract, the MTT assay on 3T3-L1 preadipocytes cell line was studied *in vitro,* and it was found that the methanol extract of *P. virgatus* was noncytotoxic at various concentrations as shown in Figure 10. Since the presented data clearly indicates that methanol extract showed markedly higher antioxidant and α-amylase inhibitory property, it was further subjected to preliminary GC-MS analysis.

3.7. GC-MS Analysis. Preliminary GC-MS analysis, based on retention time and molecular mass, was performed to determine the nature of phytoconstituents present in methanol extract. The GC-MS spectral results and comparison of results with library search successfully enabled the identification of seven compounds with their retention time (RT), molecular formula, molecular weight (MW), and concentration (peak area %) (Table 5). The results revealed that 2,4,5-trimethoxy propenylbenzene (27%), 11-octadecenoic acid (22.29%), 9,12-octadecadienoic acid (16.91%), and hexadecanoic acid (13.36%) were found to be the four major components in the methanol extract, whereas benzenedicarboxylic acid (7.1%), tridecyl ester (8.77%), and 6-octadecynoic acid (4.56%) were found in less amounts.

Table 5: Major constituents of *P. virgatus* methanol extract revealed via GC-MS analysis.

Peak	R.T	Compound	Molecular formula	Molecular weight	Area%
1	16.508	Benzenedicarboxylic acid (synonym: phthalic acid)	$C_{12}H_{14}O_4$	222	7.1
2	17.005	2,4,5-Trimethoxy propenyl benzene (synonym: asarone)	$C_{12}H_{16}O_3$	208	27
3	18.508	Tridecyl ester (synonym: acrylic acid)	$C_{16}H_{30}O_2$	254	8.77
4	22.643	Hexadecanoic acid (synonym: palmitic acid)	$C_{17}H_{34}O_2$	270	13.36
5	26.557	9,12-Octadecadienoic acid (synonym: linoleic acid)	$C_{19}H_{34}O_2$	294	16.91
6	26.798	11-Octadecenoic acid	$C_{19}H_{34}O_3$	294	22.29
7	27.517	6-Octadecynoic acid	$C_{19}H_{34}O_4$	294	4.56

3.8. Docking Analyses. Validation of docking protocol and the size as well as center of the coordinates of the grid was carried out in order to ensure that ligands bind to the binding pocket in the correct conformation. It was performed by redocking cocrystallized acarbose into its respective binding site within porcine pancreatic α-amylase. Redocked inhibitor was found to interact with the same amino acids of the active site as was in the crystal structure (Figure 11). The root mean square deviation (RMSD) of all atoms between these two conformations was 1.58 Å, indicating that the protocol set for molecular docking is accurate. Then, the *in silico* study was done beneath the assumption that a predicted high docking score in absolute value will be predictive of a strong inhibition of the enzyme. The results showed that all the molecules depicted by GC-MS were found to bind within the active site of porcine pancreatic α-amylase with binding free energy ranging from −3.27 to −6.11 Kcal/mol (Table 6). The docking results illustrated that 9,12-octadecadienoic acid was the most active compound, followed by 11-octadecenoic acid, asarone, and acrylic acid with binding free energy of −6.11, −5.55, −5.21, and −5.19 Kcal/mol, respectively, against α-amylase. The following amino acids: Trp57, Tyr62, Leu162, Leu165, Asp197, Lys200, His201, Glu233, and Ile235 were found to be the key residues playing important role in stabilizing the complex (Figure 12 and Table 6).

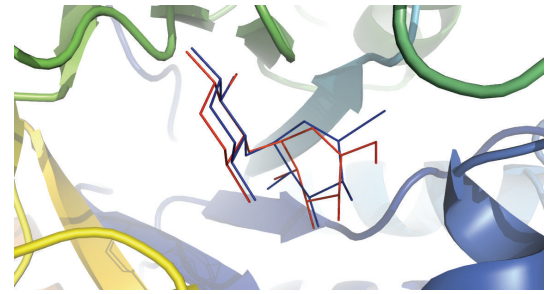

Figure 11: Binding orientation of the crystallized (red) and redocked (blue) acarbose.

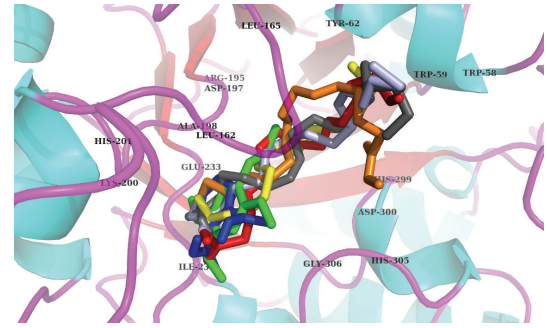

Figure 12: Binding pattern of the compounds depicted via GC-MS analyses within the active site of pancreatic α-amylase.

4. Discussion

Currently used synthetic drugs, which are known to protect against type 2 DM and oxidative damage, have their adverse side effects. As a result, consumption of natural antioxidants, that are known to be effective scavengers of free radicals, through plants, food, or dietary supplements, interrupts the production of ROS and thus helping in the prevention of various diseases including type 2 DM [4, 8, 9, 33, 34]. In the current study, a sequential extraction involving the solvent of decreasing polarity to extract the bioactive compounds was used because the nature, polarity, and hence the solubility of the bioactive compounds in *P. virgatus* were unknown. Our data initially indicated that methanol extract of *P. virgatus* not only showed significant presence of polyphenols but also exhibited the highest amount of TPC than other extracts, which is in well agreement with earlier studies [14, 35]. Our results illustrated significantly higher total antioxidant capacity of the methanol extract of *P. virgatus*, which is even greater than the standard ascorbic acid. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and generally correlates with the presence of reductones, which have been shown to exert

Table 6: Molecular docking results of pancreatic α-amylase with different compounds of *P. virgatus* methanol extract.

Compounds	Binding energy (kcal/mol)	Residues involved
Hexadecanoic acid	−4.58	TRP-59, TYR-62, GLN-63, LEU-162, LEU-165, ADP-197, ALA-198, LYS-200, HIS-201, ILE-235, ASP-300
Asarone	−5.21	LEU-162, ASP-197, ALA-198, LYS-200, HIS-201, GLU-233, VAL-234, ILE-235
Phthalic acid	−4.00	TYR-151, ALA-198, LYS-200, HIS-201, GLU-233, ILE-235
Acrylic acid	−5.19	TRP-59, TYR-62, GLN-63, LEU-162, LEU-165, LYS-200, HIA-201, GLU-233, VL-234, ILE-235
11-Octadecenoic acid	−5.55	TRP-59, TYR-62, LEU-162, LEU-165, LYS-200, HIS-201, GLU-233, VAL-234, ILE-235
9,12-Octadecadienoic acid	−6.11	TRP-59, TYR-62, GLN-63, LEU-162, LEU-165, ALA-198, LYS-200, HIS-201, GLU-233, ILE-235
6-Octadecynoic acid	−4.92	TRP-58, TRP-59, TYR-62, HIS-101, LEU-162, VAL-163, LEU-165, ASP-197, ALA-198, LYS-200, HIS-201, GLU-233, ILE-235, HIS-305

antioxidant activity by breaking the free radical chain and donating a hydrogen atom [36].

Phenolic compounds have been said to account for most of the antioxidant activities of plant extracts [37], and thus antioxidant activity of methanol extract would be granted to these polyphenolic compounds. Our results observed high linear correlation between the FRAP value and TPC of various extracts of *P. virgatus*, indicating that the antioxidant activity of the extract is mainly due to its phenolic content.

It is well known that compounds capable of scavenging free radicals can delay, inhibit, or prevent the oxidation of various biomacromolecules and diminish the oxidative stress, which play major role in the development of several diseases like diabetes [4, 5, 10]. Our results demonstrated that *P. virgatus* methanol extract exhibited strong DPPH radical scavenging activity with respect to other extracts (Table 4), which in turn signifies its potent antioxidant activity. These results are in concordance with another study where crude methanol extract of *P. virgatus* showed higher DPPH radical scavenging activity [20].

A great number of *in vitro* experiments showed that hydroxyl radical, the most reactive among ROS, has the capacity to damage DNA, which appears to represent the major target involved in mutagenesis, carcinogenesis, diabetes, and so forth [3, 38]. Therefore, we initially evaluated the role of different extracts of *P. virgatus* in directly scavenging and in protecting the DNA damage caused by hydroxyl radicals. The degradation of deoxyribose to thiobarbituric acid reactive substances (TBARS) by hydroxyl radicals was markedly decreased by methanol and water extract. The observed IC_{50} value of *P. virgatus* methanol extract indicates that this extract is a better hydroxyl radical scavenger than standard mannitol. There are several reports indicating that various antioxidants present in plant are good scavengers of hydroxyl radicals [39], including only one report from *Phyllanthus* sp., that is maderaspatensis [17].

Damage of plasmid DNA due to $OH^{\cdot}$ radical resulted in single- and double-strand break. Studies have identified potent antioxidants from plants that are effective against DNA damage [38]. *P. virgatus* was also known for its potent antioxidant property [20], and our preliminary result showed that the DNA damage induced by hydroxyl radical was significantly ameliorated by *P. virgatus* methanol extract (Figure 4) and is almost comparable with standard mannitol. The above action of *P. virgatus* methanol extract was may be due to its strong antioxidant activity, which prevents the reaction of Fe^{2+} ions with H_2O_2 or through mechanism including quenching of ROS by donating H atom. The result illustrating the potent oxidative DNA damage protective activity of methanol extract is well correlated with results of our various antioxidant parameters, illustrating the greater ROS-quenching capacity by methanol extract, which in turn indicates that this extract may be used as therapeutic agent in treating ROS-related pathological conditions including type 2 DM.

There are several therapeutic approaches to decrease postprandial hyperglycemia; one of which is retarding the absorption of glucose through inhibition of carbohydrate-hydrolyzing enzymes either α-amylase or α-glucosidase [6–8]. Presently used oral antihyperglycemic agents have however a risk of inducing hypoglycemia and lose their efficacy over time, and they have prominent side effects and fail to significantly alter the course of diabetic complications [7]. The management of diabetes without any side effects is still a challenge; therefore, the World Health Organization (WHO) has recommended research and use of complementary medicines from plants for the treatment and management of this disease [40].

Our investigation provides the first evidence of α-amylase inhibitory property of sequentially extracted *P. virgatus*

extracts. The initial screening of all extracts of *P. virgatus* demonstrated some α-amylase inhibitory activity with maximum activity in methanol extract. In addition, this extract also exhibited a concentration-dependent increase in percent inhibition of α-amylase activity with an IC_{50} value of $33.20 \pm 0.556\,\mu$g/mL, which is quite better than the IC_{50} value of standard acarbose $76.88 \pm 0.277\,\mu$g/mL. This observation suggests that porcine pancreatic α-amylase is inhibited by more polar constituents of *P. virgatus*, which is in agreement with another study that reported α-amylase inhibitory activities in the more polar extracts of plant materials [41, 42]. Thus, the enzyme inhibitory activity of methanol extracts could be due to the presence of polyphenols, flavonoids, and their glycosides, which are known to be soluble in more polar solvents.

Enzymes inhibitors obeying Michaelis-Menten kinetics are often characterized in terms of their effects on the kinetic constants, K_m and V_{max}, using either Lineweaver-Burk plots or Dixon secondary plots. In the current study, *P. virgatus* methanol extracts demonstrated noncovalent type of noncompetitive (V_{max} decreased whereas K_m remained the same) mode of inhibition against porcine pancreatic α-amylase, whereas acarbose was competitive in nature. These observations might suggest that the α-amylase inhibitory components of methanol extracts do not resemble the normal substrates of the enzymes in structure [29]. Further, the mechanism of inhibition of acarbose seems to be due to the unsaturated cyclohexene ring and the glycosidic nitrogen linkage that mimics the transition state for the enzymatic cleavage of glycosidic linkages [43]. It has been previously shown that acarbose is a competitive inhibitor of α-amylase, which is in strong agreement with our results [29].

Moreover, the strong correlation demonstrated between total antioxidant and polyphenol contents and between DPPH radical scavenging and α-amylase activity of methanol extracts suggests that polyphenol compounds involved in total antioxidant/antiradical activity may also is directly or indirectly intervene in α-amylase inhibitory activity. Postprandial blood glucose level is known to be regulated by glucose uptake, a rate limiting step for glucose metabolism. In the present study, we used differentiated 3T3-L1 cell lines because it was previously established that glucose uptake was higher in these cells than in undifferentiated one, which is probably due to the presence of glucose transporter-4 (GLUT4) in their expression [44].

Our result, for the first time, speculated that *P. virgatus* methanol extract possesses the ability to improve glucose uptake in the adipose tissue. The positive controls chosen for glucose uptake in 3T3-L1 cells due to their antidiabetic activity were metformin and insulin, as they are known specifically for the affirmative effect on the translocation of GLUT4 to the cell surface thereby promoting glucose uptake. Insulin and metformin significantly promote the glucose uptake alone, while there is almost a negligible synergistic effect when given in combination (in presence or absence of extract). On this basis, a mechanism of action of *P. virgatus* may be hypothesized, which could be linked to insulin-mediated glucose transport transduction pathway in which a series of proteins (phosphatidylinositol-3 kinase, protein kinase C, and PPAR) are involved [44, 45]. This may perhaps lead to the translocation of GLUT4 to the plasma membrane to facilitate the uptake of glucose from the bloodstream into the cells. Thus, the occurrence of polyphenolic compounds in methanol extract may be responsible for the activation of these signaling proteins [11, 46, 47] and might therefore also account for their upregulation of these proteins, which in turn is responsible for its glucose uptake activity.

In a search for the source of bioactive compounds responsible for the aforementioned actions of *P. virgatus* methanol extract, preliminary GC-MS analysis was performed. For the first time, it was noted that the sequentially extracted *P. virgatus* methanol extract contains phthalic acid, asarone, acrylic acid, palmitic acid, linoleic acid, 11-octadecenoic acid, and 6-octadecynoic acid (Table 5). Various species of *Phyllanthus* reported the presence of these compounds with antioxidant and antidiabetic activity [13, 48, 49].

From the above data, it may be concluded that these bioactive compounds of *P. virgatus* methanol extract alone or in combination possess significant antioxidant activity, which could be responsible in ameliorating all the above oxidative damages, including inhibition of amylase activity. However, our *in silico* investigation is a novel approach to identify the molecular targets involved in inhibition of α-amylase activity by this extract. We previously revealed the implication of molecular docking studies in elucidating the mechanistic aspect of natural products against different enzymes [50]. Molecular simulation study is considered to be an important vehicle to investigate the mode of interaction of ligand against its target protein that also makes us understand their binding or inhibition mechanism. Validation of docking protocol was performed by redocking cocrystallized acarbose into its respective binding site within porcine pancreatic α-amylase. Redocked inhibitor was found to interact with the same amino acids of the active site as was in the crystal structure with RMSD of 1.58 Å between these two conformations (Figure 11). Our results demonstrated that 9,12-octadecadienoic acid (linoleic acid) was the most potent inhibitor of pancreatic α-amylase, whereas 11-octadecenoic acid, 2,4,5-trimethoxy propenyl (asarone), and tridecyl ester also showed good inhibitory activity in terms of their binding energy. These results are well supported by wet lab studies where asarone, linoleic acid, and acrylic acid have been reported to exhibit the antidiabetic property [50, 51]. Of the catalytic triad [52], Asp197 and Glu233 were found to be very much involved in the positioning of inhibitor within the active sites of pancreatic α-amylase. Our results are in general agreement with the mechanism of action proposed for acarbose [8], since they showed for α-amylase that active ligands interact with the side chains of Asp197, Glu233, and Asp300 (Figure 12, Table 6). All the inhibitors were anchored at the catalytic center, which might explain why the enzymatic activity of α-amylase was successfully blocked. Thus, it is very difficult to name a single compound responsible for the whole activity. Therefore, based on our *in vitro* and *in silico* results, we suggest that the α-amylase inhibitory activity of *P. virgatus* methanol extract might be because of the synergistic effect of these compounds.

5. Conclusion

In conclusion, the results for the first time demonstrated a strong antioxidant and α-amylase inhibitory as well as glucose uptake property of sequentially extracted *P. virgatus* methanol fraction, compared to other extracts. The docking studies further confirmed the antidiabetic property of the bioactive compounds revealed via GC-MS analysis of this extract and suggested that α-amylase inhibitory property of this extract was maybe due to the synergistic effect of these bioactive compounds. Thus, it is a good approach to manage type 2 DM as a whole with these compounds/extracts, which showed good enzyme inhibitory and antioxidant activities. Further, a thorough and full-fledged *in vivo* study is needed to explore the role of these extracts and also their bioactive compounds.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Characterization of Dendritic Cell and Regulatory T Cell Functions against *Mycobacterium tuberculosis* Infection

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Glutathione (GSH) is a tripeptide that regulates intracellular redox and other vital aspects of cellular functions. GSH plays a major role in enhancing the immune system. Dendritic cells (DCs) are potent antigen presenting cells that participate in both innate and acquired immune responses against microbial infections. Regulatory T cells (Tregs) play a significant role in immune homeostasis. In this study, we investigated the effects of GSH in enhancing the innate and adaptive immune functions of DCs against *Mycobacterium tuberculosis* (*M. tb*) infection. We also characterized the functions of the sub-populations of CD4+T cells such as Tregs and non-Tregs in modulating the ability of monocytes to control the intracellular *M. tb* infection. Our results indicate that GSH by its direct antimycobacterial activity inhibits the growth of intracellular *M. tb* inside DCs. GSH also increases the expressions of costimulatory molecules such as HLA-DR, CD80 and CD86 on the cell surface of DCs. Furthermore, GSH-enhanced DCs induced a higher level of T-cell proliferation. We also observed that enhancing the levels of GSH in Tregs resulted in downregulation in the levels of IL-10 and TGF-β and reduction in the fold growth of *M. tb* inside monocytes. Our studies demonstrate novel regulatory mechanisms that favor both innate and adaptive control of *M. tb* infection.

1. Introduction

M. tb, the causative agent for tuberculosis (TB), is a slow-growing, obligate aerobic bacterium, that is, transmissible through aerosolized droplets containing as few as 2-3 bacilli [1, 2]. Although appearing as a disease of past generations, *M. tb* infects one-third of the world's population [1, 2]. In this population, two billion people are asymptomatic carriers of the bacterial infection. According to WHO, eight million infected individuals will develop active TB and two million will die per year [3]. Of the two billion individuals latently infected with *M. tb*, 10% will undergo reactivation of *M. tb* infection leading to active disease whenever their immune system gets compromised due to ageing, corticosteroid treatment, or most commonly, coinfection with human immunodeficiency virus (HIV) [4]. *M. tb* can resist complete clearance by the host immune system due to several factors including its ability to persist and remain in a dormant state in antigen presenting cells (APCs) for a lengthy duration of time [3].

GSH is a tripeptide that regulates intracellular redox and other important aspects of cell physiology [5]. GSH plays a major role in enhancing the functions of immune cells. GSH is essential for cellular homeostasis and plays a vital role in diverse cellular functions. GSH synthesis occurs within cells in two closely linked, enzymatically controlled reactions

with the availability of cysteine usually being the rate-limiting factor [5]. In healthy cells more than 90% of the total GSH pool is in the reduced form (GSH or *r*GSH) and less than 10% exists in the oxidized disulfide form (GSSG). Among the two forms, *r*GSH is considered to be the functional form having the antioxidant and other immune enhancing properties [6].

The two major layers of defense against *M. tb* infection include preformed innate immunity that lacks specificity and a highly specific and effective adaptive immunity. DCs are potent APCs, linking the innate and adaptive immune responses [7]. DCs have the unique ability to migrate from the site of infection to a draining lymph node and subsequently recruit T cells to the site of infection thereby effectively activating the acquired immune response [8]. In addition, DCs carry a myriad of functions ranging from influencing different lymphocytes such as B cells, natural killer (NK) cells, and natural killer T (NKT) cells to initiating different T lymphocyte responses such as Th1/Th2, regulatory T cells and peripheral T cell deletion [8].

Regulatory T cells (Tregs) are critical for the maintenance of immune cell homeostasis as evidenced by the catastrophic consequences of genetic or physical ablation of the Treg population [9]. Specifically, Treg cells maintain order in the immune system by enforcing a dominant negative regulation on other immune cells [9].

In this study, we tested the effects of GSH in regulating the functions of DCs to control *M. tb* infection by performing *in vitro* studies using isolated cells from healthy subjects. The production of IL-10 and IL-12 from DCs was also analyzed to indirectly elucidate whether a cell-mediated immunity (IL-12) or an antibody-mediated (IL-10) response is produced when DCs were treated with GSH-enhancing agents such as N-acetyl cysteine (NAC, a GSH precursor) and liposomal glutathione (L-GSH). Increased production of IL-12 accompanied by Th1 CD4 T-cell responses is considered crucial for controlling *M. tb* infection. We also examined the ability of GSH to enhance the expressions of costimulatory molecules on the cell surface of DCs and to induce proliferation of T-cells.

Additionally, we characterized the effects of GSH in modulating the functions of both Tregs and non-T regs (subpopulations of CD4+ T cells) to control *M. tb* infection inside monocytes. Finally, we investigated whether increasing GSH concentrations in Tregs would result in downregulation of TGF-β and IL-10 levels leading to improved control of *M. tb* infection inside monocytes.

Considering DCs crucial role in recruiting T cells to aid in infection, we show that by increasing the intracellular levels of GSH, DC performance is enhanced in its innate function of inhibiting the intracellular growth of *M. tb* as well as its adaptive immune role as professional APCs. Furthermore, we also show that enhancing GSH in Tregs resulted in downregulation in the levels of TGF-β and IL-10 leading to better control of *M. tb* infection inside monocytes. Our results signify the importance of GSH in enhancing both the innate and adaptive immune responses against *M. tb* infection.

2. Materials and Methods

2.1. Statement of Ethics. All studies were approved by both the Institutional Review Board and the Institutional biosafety committee of the Western University of Health Sciences. All study participants were above the legal age of consent at the time of participation and written informed consent was obtained from all volunteers prior to participation in the study.

2.2. Subjects. Healthy subjects without HIV infection or a history of TB were recruited from the faculty and staff of Western University of Health Sciences. Thirty-five milliliters (mL) of blood was drawn once from both healthy volunteers after obtaining signed informed consent.

2.3. Isolation of Monocytes and In Vitro Culture for Differentiation into DCs. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy individuals by density gradient centrifugation using ficoll histopaque, a high density pH neutral polysaccharide solution (Sigma). Monocytes were isolated from PBMCs as follows: PBMCs (1×10^5 cells/well) were distributed in poly-L lysine (0.005%, Electron Microscopy Sciences) treated 96-well tissue culture plates, and incubated overnight at 37°C to facilitate monocyte adherence. Following overnight adherence, the nonadherent cells were removed and fresh Roswell Park Memorial Institute media (RPMI (Sigma)) supplemented with 5% human AB serum (Sigma), 2 mM glutamine, 20 ng/mL GM-CSF (Ebioscience), and 40 ng/mL IL-4 (Ebioscience) was added to the adherent monocytes which were then incubated for 7 days to allow monocytes to differentiate into DCs.

2.4. Preparation of Bacterial Cells for DC Infection. All infection studies were performed using the virulent laboratory strain of *M. tb*, H37Rv inside the biosafety level 3 (BSL-3) facility. *M. tb* was processed for infection as follows: static cultures of H37Rv at their peak logarithmic phase of growth (between 0.5 and 0.8 at A600) were used for infection of DCs. The bacterial suspension was washed and resuspended in RPMI (Sigma) containing AB serum (Sigma). Bacterial clumps were disaggregated by vortexing five times with 3 mm sterile glass beads. The bacterial suspension was passed through a 5 μm syringe filter (Millipore) to remove any existing clumps. The total number of organisms in the suspension was ascertained by plating. Processed H37Rv was frozen as stocks at −80°C. At the time of infection, frozen stocks of processed H37Rv were thawed and used for infection of DCs and monocytes.

2.5. Assay of GSH Levels in DCs from Healthy Subjects. We determined the effects of *M. tb* infection in decreasing the levels of total GSH in isolated DCs from healthy subjects by spectrophotometry using an assay kit from Arbor Assays. Furthermore, we also tested the ability of GSH-enhancing agents such as NAC and L-GSH in restoring the levels of total GSH and the ratio of *r*GSH versus GSSG in *M. tb*-infected DCs. Monocyte derived DCs were infected with processed

H37Rv at a multiplicity of infection of 1:1 (1 bacterium for every single DC) and incubated for 2 hours to allow for phagocytosis. Unphagocytosed bacteria were removed by washing the infected DCs three times with warm sterile PBS. Infected DCs were cultured in RPMI + 5% AB serum at 37°C + 5% CO_2 in the presence and absence of NAC (10 and 20 mM) or L-GSH (10 and 20 μM, Your Energy Systems, LLC Palo Alto, CA, USA. The liposomal formulation of GSH (L-GSH) used in these studies is a proprietary product of Your Energy Systems (YES), LLC (Palo Alto, CA, USA) called ReadiSorb. Infected DCs were terminated at 5 days after infection to determine the intracellular levels of GSH. Uninfected DCs maintained in culture for 5 days served as controls for baseline levels of GSH. Briefly, DCs (3×10^5) were washed and resuspended in ice cold 5% 5-sulfo-salicylic acid dehydrate solution (MP Biomedicals). Supernatants collected after centrifugations were analyzed for total GSH and oxidized GSH (GSSG) as per manufacturer's instructions. *r*GSH was calculated by subtracting measured GSSG concentrations from the measured total GSH concentrations. All GSH measurements were normalized with total protein levels.

2.6. Assay of Total Protein Levels in Lysates. Proteins in the isolated cell lysates were measured by Bradford's method [10] using a Coomassie protein assay reagent (Thermo Scientific). This assay helped to determine the amount of protein in each well. By dividing the total protein value from the GSH assay, it helped to confirm that the variation of GSH is due to the treatments added.

2.7. Intracellular Survival of H37Rv in GSH-Enhanced DCs. To examine the role of DCs and GSH in innate defense against *M. tb* infection, we determined the intracellular survival of *M. tb* inside DCs that were cultured in the presence and absence of GSH-enhancing agents. DCs were infected with processed H37Rv at a low dose multiplicity of infection of 1:10 (1 bacterium for every 10 DCs) and incubated for 2 hours to allow for phagocytosis. Unphagocytosed mycobacteria were removed by washing the infected DCs three times with warm sterile PBS. Infected DCs were cultured in RPMI + 5% AB serum at 37°C + 5% CO_2 in the presence and absence of NAC (5, 10, and 20 mM), L-GSH (5, 10, and 20 μM, Your Energy Systems, LLC Palo Alto, CA, USA), or buthionine sulfoximine (BSO-500 μM). BSO inhibits the synthesis of GSH by inhibiting the activity of the rate limiting step enzyme that is involved in the synthesis of GSH leading to decreased intracellular levels of GSH. H37Rv-infected DCs that were sham-treated (RPMI + 5% AB serum) served as a control. Infected DCs were terminated at 1 hour and 5 days after infection to determine the intracellular survival of H37Rv.

2.8. Termination of H37Rv-Infected DC Cultures and Measurement of Colony Forming Units (CFUs). Termination of H37Rv-infected DCs was performed by the addition of 200 μL of sterile distilled water to each culture well. The collected DC lysates were diluted in sterile water and plated on 7H11 medium (Hi Media) enriched with albumin dextrose complex (ADC), to estimate the extent of H37Rv growth in DCs by counting the CFUs.

2.9. Assay of IL-1, IL-12, and IL-10 in DC Supernatants. Levels of IL-1, IL-12 and IL-10 in DC supernatants were measured by enzyme linked immunosorbent assay (ELISA) (eBioscience).

2.10. Immunocytochemical Analysis of Costimulatory Markers Expressions on the Cell Surface of DCs. The effects of GSH-enhancing agents in augmenting the expressions of costimulatory molecules on the cell surface of DCs were determined by immuno-fluorescent staining. DCs (both uninfected (control category) and H37Rv-infected) were incubated for 5 days on sterile glass cover slips in the presence and absence of GSH-enhancing agents. DCs were fixed with 3.8% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 30 minutes. Fixed DCs were washed twice for 5 minutes in ice cold PBS and then blocked for 30 minutes in 1% bovine serum albumin (BSA) in PBS + 0.2% Tween-20 (PBST). Blocked DCs were incubated with mouse anti-human HLA-DR IgG, mouse anti-human CD80 IgG, and mouse anti-human CD86 IgG (1 μg/mL each, Ebioscience) in PBST, overnight at 4°C with mild shaking. Antibodies against HLA-DR and CD80 were labeled with FITC 488 (green) whereas antibodies against CD86 were labeled with PE 546 (red). After incubation with fluorescent labeled antibodies, stained DCs were washed three times for 5 minutes with PBS to remove excess stain. A single drop of mounting media containing 4′, 6-diamidino-2-phenylindole (DAPI) was placed on glass slides before inverting the glass cover slips with attached, stained DCs and placing them on the slide. The cover slips were then sealed to the glass slide using nail polish around the edges of the cover slip. The stained slides were then viewed using an inverted fluorescent microscope. Images were obtained using an integrated digital camera. Images were subsequently analyzed using ImageJ, a free software program available from the National Institutes of Health (http://rsbweb.nih.gov/ij/). Correcting for background fluorescence, average fluorescent intensity was measured for each labeled protein.

2.11. Intracellular Survival of H37Rv in Matured DCs. Lipopolysaccharide (LPS), an activating agent is also known to induce maturation of DCs. We therefore tested the effects of GSH in improving the control of *M. tb* infection inside mature DCs by determining the intracellular survival of H37Rv in GSH-enhanced and LPS-treated DCs. Monocyte derived DCs were infected with processed H37Rv at a multiplicity of infection of 1:1 (1 bacterium for every single DC) and incubated for 2 hours to allow for phagocytosis. Unphagocytosed mycobacteria were removed by washing and infected DCs were cultured in medium containing LPS (1 μg/mL) in the presence and absence of NAC (5, 10, and 20 mM), L-GSH (5, 10, and 20 μM), or BSO (500 μM). Infected DCs were terminated at 1 hour and 5 days after infection to determine the intracellular survival of H37Rv in mature DCs. H37Rv-infected + LPS-treated DCs served as controls for GSH-enhancing agent treatment groups.

2.12. DCs and T Cell Proliferation Assays. We determined the ability of GSH-enhanced DCs to induce proliferation of T cells by quantifying the fluorescence intensity of carboxyfluorescein diacetate, succinimidyl ester (CFSE). DCs were treated overnight with NAC (10 mM) either alone or in combination with NAC + LPS (1 μg/mL). Following overnight incubation with NAC, DCs were infected with H37Rv, washed, and resuspended in fresh media containing no additives. Allogeneic T cells were isolated from PBMCs using a nylon wool column (Polysciences). Isolated T cells were washed two times in PBS to remove any serum. T cells were resuspended in PBS at a cell density of $5–10 \times 10^6$/mL. CFSE, at a concentration of 1 μM, was added to allogeneic T cell suspension, mixed immediately and incubated for 10 minutes at room temperature in the dark. Labeling was stopped by the addition of 4-5 volumes of cold complete media followed by incubation on ice for 5 minutes. T cells were then washed three times with complete media and evenly distributed to the wells containing infected DCs. CFSE allows for cell division tracking of T cells, that is, the tracking of the proliferation of T cells upon the coincubation with H37Rv-infected and NAC treated DCs. In separate studies, GSH-levels in T cells (not DCs) were manipulated by overnight treatment with different concentrations of NAC (5, 10 and 20 mM). Following overnight incubation with NAC, T cells were washed three times with PBS, labeled with CFSE and then added to the infected DCs to determine the ability of GSH-enhanced T cells to effectively respond to the *M. tb*-infected DCs and proliferate. Seven days after incubation, T cells from both the studies (GSH-altered DCs and GSH-altered T cells) were aspirated and fixed in PFA and analyzed for T cell proliferation using flow cytometry outside of BSL-3 lab.

2.13. Determination of Intracellular Viability of H37Rv in Cocultures of Infected Monocytes and CD4+ T Cells (Non-T regs and Tregs). We characterized the roles of sub-populations of CD4+ T cells, specifically Tregs (CD4+CD25+ T-cells) and non-T regs (CD4+CD25− T cells), in regulating the host immune responses against *M. tb* infection by quantifying the intracellular viability of H37Rv inside monocytes that were cultured in the presence and absence of Tregs and non-Tregs. Tregs and non-Tregs were isolated from PBMCs derived from healthy subjects using midi-MACS LD columns and mini-MACS MS columns from Miltenyi Biotech. This method of isolation resulted in 99% pure population of Tregs and non-Tregs [11]. Adherent monocytes were infected with processed H37Rv at a multiplicity of infection of 1 : 1 and incubated for 2 hours for phagocytosis. Unphagocytosed mycobacteria were removed by washing the infected monocytes three times with sterile PBS. Infected monocytes were cultured in RPMI containing 5% AB serum in presence and absence of autologous Tregs and non-Treg cells. Prior to co-incubation with infected-monocytes, autologous CD4+ T cells (both Tregs and non-Tregs) were incubated overnight with NAC (10 mM), washed with PBS, resuspended in fresh RPMI containing AB serum (without any stimulants), and then added to the infected monocytes (monocyte: T cell ratio was adjusted to 1 : 1). H37Rv-infected monocytes cultured in the absence of CD4+ T cells (Tregs and non-Tregs) served as controls. Infected monocyte T cell cocultures were terminated at 1 hour and 5 days after infection to determine the intracellular survival of H37Rv.

2.14. Termination of M. tb-Infected Monocytes-Autologous CD4+ T Cell Cocultures. H37Rv-infected monocytes cultured in the presence and absence of Tregs and non Tregs were terminated at 1 hour and 5 days after infection. During termination, supernatants were removed and adherent monocytes were lysed by addition of 200 μL sterile cold distilled water. 25 μL of 10-fold diluted lysates were plated on 7H11 medium (Hi Media) enriched with ADC, to estimate the extent of H37Rv growth or killing in co-cultures of monocytes and CD4+ T cells. Cell-free supernatants were plated to determine extracellular H37Rv growth. Cell-free supernatants were also used for determining the levels of IL-10, and TGF-β.

2.15. Assay of IL-10 and TGF-β in Supernatants from Cocultures of Monocytes and CD4+ T Cells. Cytokines (IL-10 and TGF-β) were measured in supernatants from co-cultures of monocytes and CD4+ T cells (Tregs and non Tregs) by ELISA (eBioscience).

2.16. Statistical Analysis. All statistical analysis was done using GraphPad Prism6 software on the mean ± standard error for $n = 5$ individuals, unless otherwise indicated. Results were considered significant for $P \leq 0.05$.

3. Results

3.1. GSH Measurement in Lysates from DCs. Infection of DCs with H37Rv resulted in 50% decrease in the intracellular levels of total GSH compared to uninfected DCs (Figure 1(a)). Treatment of H37Rv-infected DCs with 10 mM NAC resulted in restoration of GSH to similar levels as uninfected DCs (Figure 1(a)). Maximum enhancement in the intracellular levels of both total and *r*GSH was observed when H37Rv-infected DCs were treated with 20 mM NAC (8-fold increase) or 20 μM L-GSH (3-fold increase) (Figures 1(a), 1(b), 1(c) and 1(d)). We also compared the percentage of GGSG and *r*GSH for various treatment conditions and we observed that treatment of H37Rv-infected DCs with 20 μM L-GSH resulted in 99% *r*GSH and just 1% GSSG highlighting the effective restorative effects of L-GSH at 1000-fold lower concentration compared to NAC.

3.2. Intracellular Survival of H37Rv in DCs. In our low-dose *M. tb* infection studies, we observed a twentyfold increase in the intracellular growth of H37Rv in unstimulated DCs between the initial and final time points of termination (Figure 2). Treatment of DCs with GSH-enhancing agents such as NAC (5 mM and 10 mM) or L-GSH (5 μM, 10 μM, and 20 μM) resulted in reduction in the fold increase in the growth of *M. tb* inside DCs (Figure 2). Among various concentration of NAC that were tested, maximum reduction

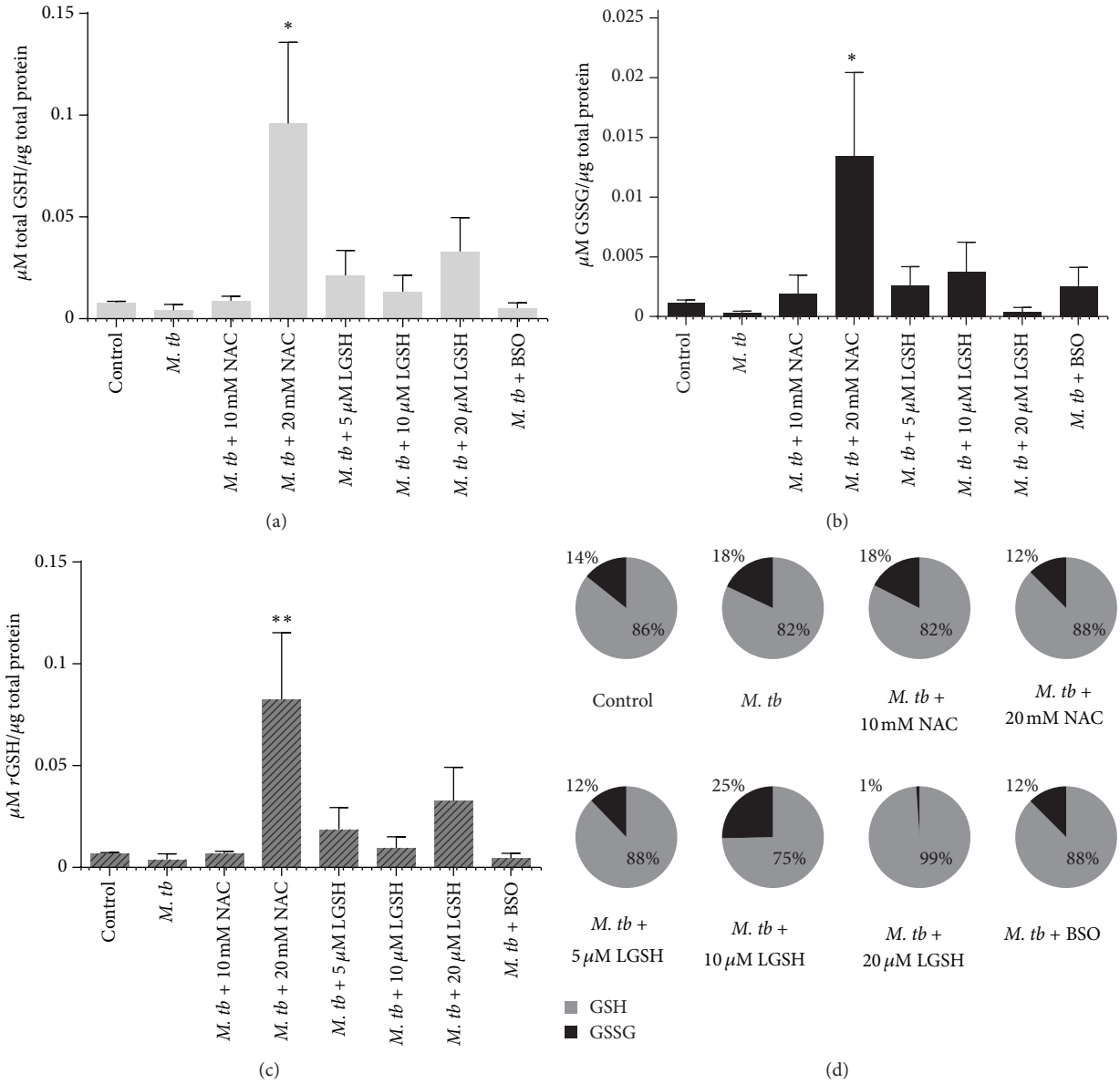

Figure 1: GSH measurements in DCs infected with H37Rv and treated with NAC, L-GSH, or BSO. GSH levels were measured in isolated DCs from healthy subjects that were infected *in vitro* with H37Rv and treated with various stimulants such as NAC, L-GSH, and BSO, by spectrophotometry using an assay kit from Arbor Assays. All GSH measurements were normalized with total protein levels. Proteins in the cell lysates of DCs were measured by Bradford's method using a Coomassie protein assay reagent. Results shown are for $n = 3$ individuals and analyzed for significance by ANOVA, Dunnett's multiple comparisons test, comparing all treatment categories to the infected-untreated control. (a) Total GSH (*r*GSH + GSSG), $^{*}P \leq 0.05$. (b) GSSG, $^{*}P \leq 0.05$. (c) *r*GSH was calculated by subtracting measured GSSG concentrations from the measured total GSH concentrations, $^{**}P \leq 0.01$. (d) Composition of total GSH, represented in percentages of *r*GSH and GSSG.

in the fold-growth of *M. tb* was observed when DCs were treated with 5 mM NAC (only tenfold increase in the growth of H37Rv compared to unstimulated DCs in which there was twentyfold increase in the growth of *M. tb*). Similarly, treatment of H37Rv-infected DCs with 5 μM concentration of L-GSH (Figure 2) resulted in utmost reduction in the fold-growth of H37Rv (there was only ninefold increase in the intracellular growth of H37Rv between the initial and final time point of termination). Although treatment with 20 mM NAC resulted in maximum enhancement in the intracellular levels of GSH in DCs, we did not however observe any decrease in the fold growth of *M. tb* under these conditions indicating the need for maintaining optimum levels of GSH in the intracellular environment in order to effectively inhibit the growth of *M. tb* (Figure 2).

3.3. IL-1 Assay in DC Supernatants. H37Rv infection of DCs resulted in 2.5-fold increase in the production of IL-1

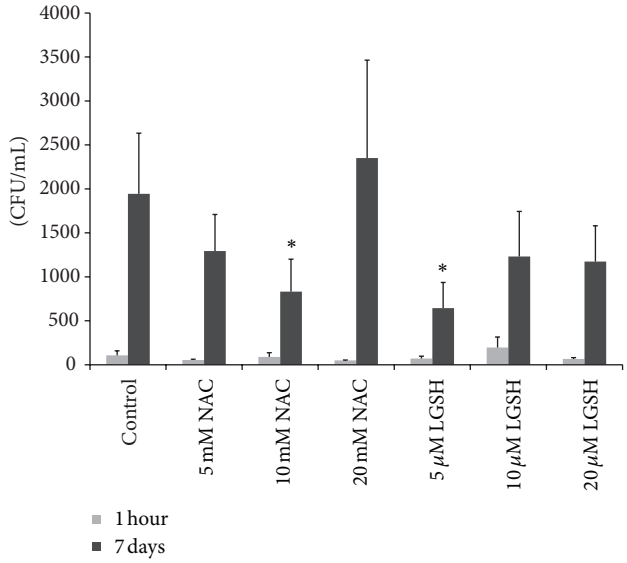

Figure 2: Intracellular survival of H37Rv in DCs treated with NAC and L-GSH. Lysates from H37Rv infected DCs were plated on 7H11 agar and colonies were counted. Results were analyzed by ANOVA with Dunnett's multiple comparisons test, comparing all treatment categories to the infected-untreated control, $^{*}P \leq 0.05$.

compared to uninfected DCs (Figure 3(a)). Enhancing the levels of GSH in DCs caused decrease in the production of IL-1. Specifically, treatment of DCs with 10 mM NAC resulted in undetectable levels of IL-1 (Figure 3(a)). Similarly treatment of H37Rv-infected DCs with 5 μM L-GSH resulted in a notable decrease in the levels of IL-1 compared to other concentrations of L-GSH used in the treatment of H37Rv-infected DCs (Figure 3(a)).

3.4. Assay of IL-12 and IL-10 in Supernatants from DCs. Treatment of H37Rv-infected DCs with L-GSH (5 μM) resulted in increased production of IL-12, a Th1 polarizing cytokine (Figure 3(b)). H37Rv infection of DCs resulted in 3-fold increase in the production of IL-10 compared to uninfected DCs (Figure 3(c)). Treatment of H37Rv-infected DCs with either NAC (10 mM) or L-GSH (5 μM) resulted in decreased production of IL-10, a Th2 polarizing cytokine (Figure 3(c)). Decreased IL-10 may favor a Th1 response.

3.5. Assay of DC Cell Surface Markers. We examined the effects of GSH in increasing the expressions of costimulatory molecules on the cell surface *M. tb*-infected DCs by immunofluorescence staining. H37Rv infection of DCs resulted in 2-fold increase in the expression of CD80 compared to uninfected control DCs. We observed that treatment of H37Rv-infected DCs with 10 mM NAC and 20 μM L-GSH resulted in 2.5-fold increase in the expression of CD80 compared to H37Rv-infected DCs and 5-fold increase in comparison to uninfected control category. Consistent with our observations on the CD80 marker, we also found a similar trend with the expression of CD86 marker; that is, treatment of H37Rv-infected DCs with 10 mM NAC and 20 μM L-GSH resulted in 2.5-fold increase in the expression of CD86 compared to H37Rv-infected DCs and 5-fold increase in comparison to uninfected control. Importantly, L-GSH added at 500x lower concentration compared to NAC is still able to induce the upregulation of both CD80 and CD86 molecules (Figures 4(a) and 4(b)). We observed a noticeable increase in the expression of HLA-DR only when the H37Rv-infected DCs were treated with 10 μM L-GSH (4-fold increase) and 20 μM L-GSH (5-fold increase) (Figure 4(c)). Our results signify the importance of GSH in enhancing the expressions of HLA-DR, CD80 and CD86 on the cell surface of *M. tb*-infected DCs.

3.6. Determination of Intracellular Viability of H37Rv in LPS-Treated DCs. We tested the effects of GSH-enhancing agents (NAC/L-GSH) in improving the control of *M. tb* inside matured DCs. Maturation of DCs was induced by treatment with LPS. We observed an 8-fold increase in the intracellular growth of H37Rv in LPS-treated DCs between the initial and final time point of termination (Figure 5). Treatment of H37Rv-infected DCs with a combination of LPS + NAC (20 mM) resulted in effective inhibition in the growth of intracellular H37Rv (there was only 2-fold increase in the growth of H37Rv). Treatment of H37Rv-infected DCs with a combination of LPS + L-GSH (20 μM) resulted in complete stasis in the growth of H37Rv (Figure 5). Treatment of infected DCs with BSO (a GSH synthesis inhibitor) resulted in 10-fold increase in the intracellular growth of H37Rv in DCs (Figure 5). Our results indicate that treatment of *M. tb*-infected DCs with a combination of NAC + LPS resulted in further improvement in the control of *M. tb* infection. Additionally, treatment of *M. tb*-infected DCs with a combination of L-GSH + LPS resulted in complete inhibition in the growth of intracellular *M. tb*.

3.7. Determination of Allogeneic T Cell Proliferation in Response to H37Rv-Infected DCs. We assessed the effects of GSH-enhancement in *M. tb*-infected DCs to improve their ability to induce proliferation of allogeneic T cells by CFSE staining. We observed that treatment of H37Rv-infected DCs with NAC (10 mM) resulted in a maximum increase in the proliferation index of allogeneic T-cells (Figure 6(a)). Treatment of H37Rv-infected DCs with a combination of LPS + NAC did not result in an additional increase in proliferation index of T cells. These results demonstrate the effects of GSH in enhancing the ability of *M. tb*-infected DCs to induce proliferation of T cells (Figure 6(a)).

3.8. Determination of T Cell Proliferation of GSH-Altered T Cells in Response to H37Rv-Infected DCs. We then investigated the proliferation capacity of GSH-enriched allogeneic T cells in response to *M. tb*-infected DCs. Our results indicate that treatment of allogeneic T cells with 5 mM NAC showed the highest T-cell proliferation index. 10 mM and 20 mM treated T-cells did not result in an increase in the proliferation index (Figure 6(b)).

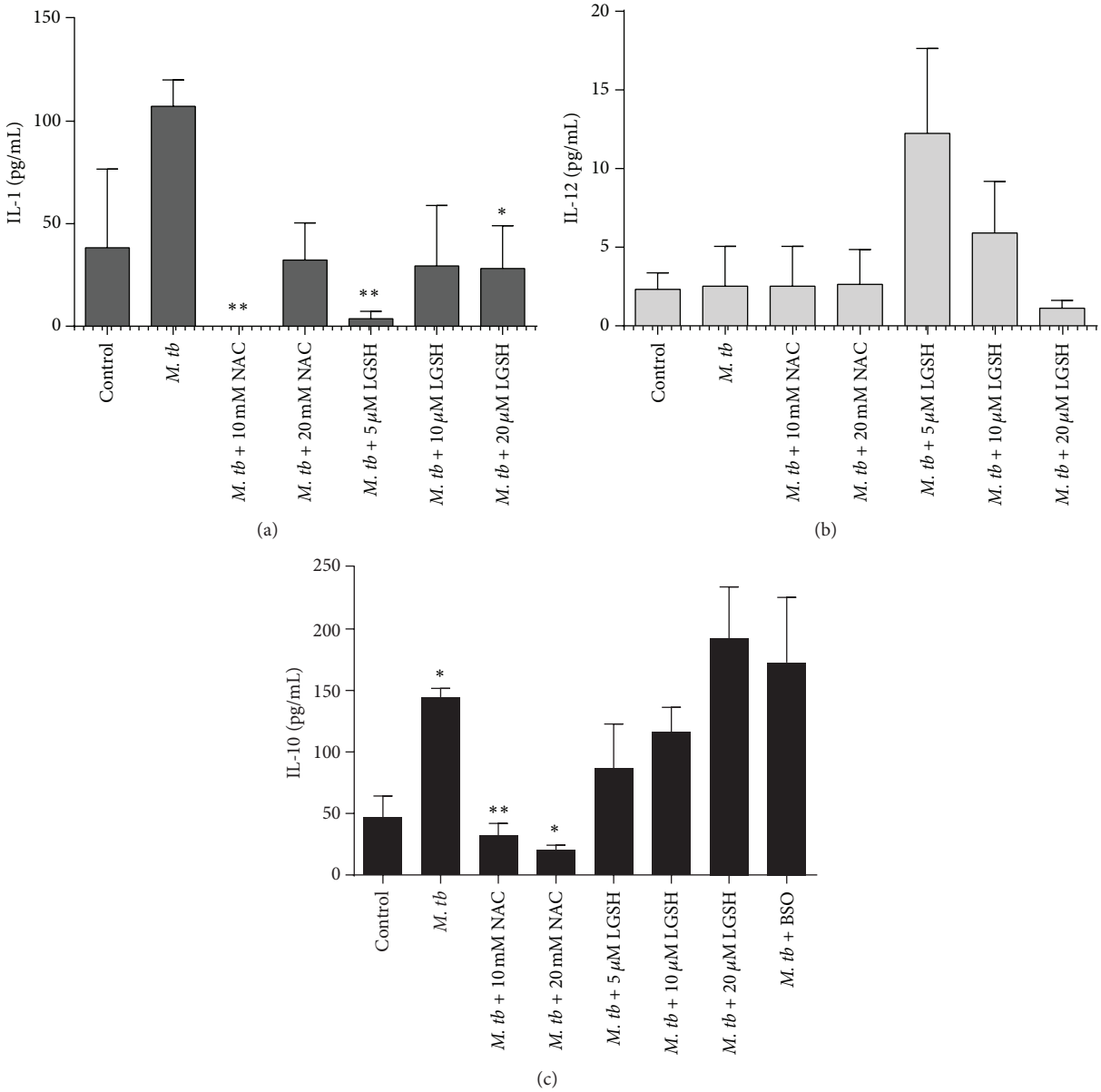

Figure 3: Cytokine measurements performed in supernatants derived from H37Rv-infected DCs. Cytokines in DC supernatants were measured on $n = 3$ samples by ELISA. Results were analyzed by ANOVA with Dunnett's multiple comparisons test, comparing all treatment categories to the infected-untreated control. Infected-untreated controls were compared to the untreated-uninfected control using the Student's t-test, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. (a) IL-1. (b) IL-12. (c) IL-10.

3.9. Determination of Intracellular Viability of H37Rv in Co-Cultures of H37Rv-Infected Monocytes and Non-Tregs. We observed a 25-fold increase in the growth of H37Rv inside human monocytes. Co-culture of H37Rv-infected monocytes with non-Treg population (CD4 T cells minus the Treg population that is, CD4+CD25− T-cells) resulted in reduction in the fold growth of H37Rv inside monocytes. This underscores the importance of CD4+CD25− non-T reg population in augmenting the capacity of monocytes to control *M. tb* infection. Treatment of non-T reg population with NAC resulted in further reduction in the CFU counts of H37Rv at 5 day time point of termination indicating improved control of *M. tb* infection when monocytes were cultured in the presence of NAC-treated non-Treg cells (Figure 7(a)).

3.10. Determination of Intracellular Viability of H37Rv in Co-Cultures of H37Rv-Infected Monocytes and Tregs. We characterized the role of Tregs in *M. tb* infection by determining the intracellular survival of H37Rv in monocytes cultured in the presence and absence of Tregs. We observed a 20-fold increase in the growth of H37Rv in monocytes that were cultured in the absence of T cells. In the presence of Tregs there was further increase in the fold growth of H37Rv inside monocytes. NAC treatment of Tregs resulted in only 12-fold

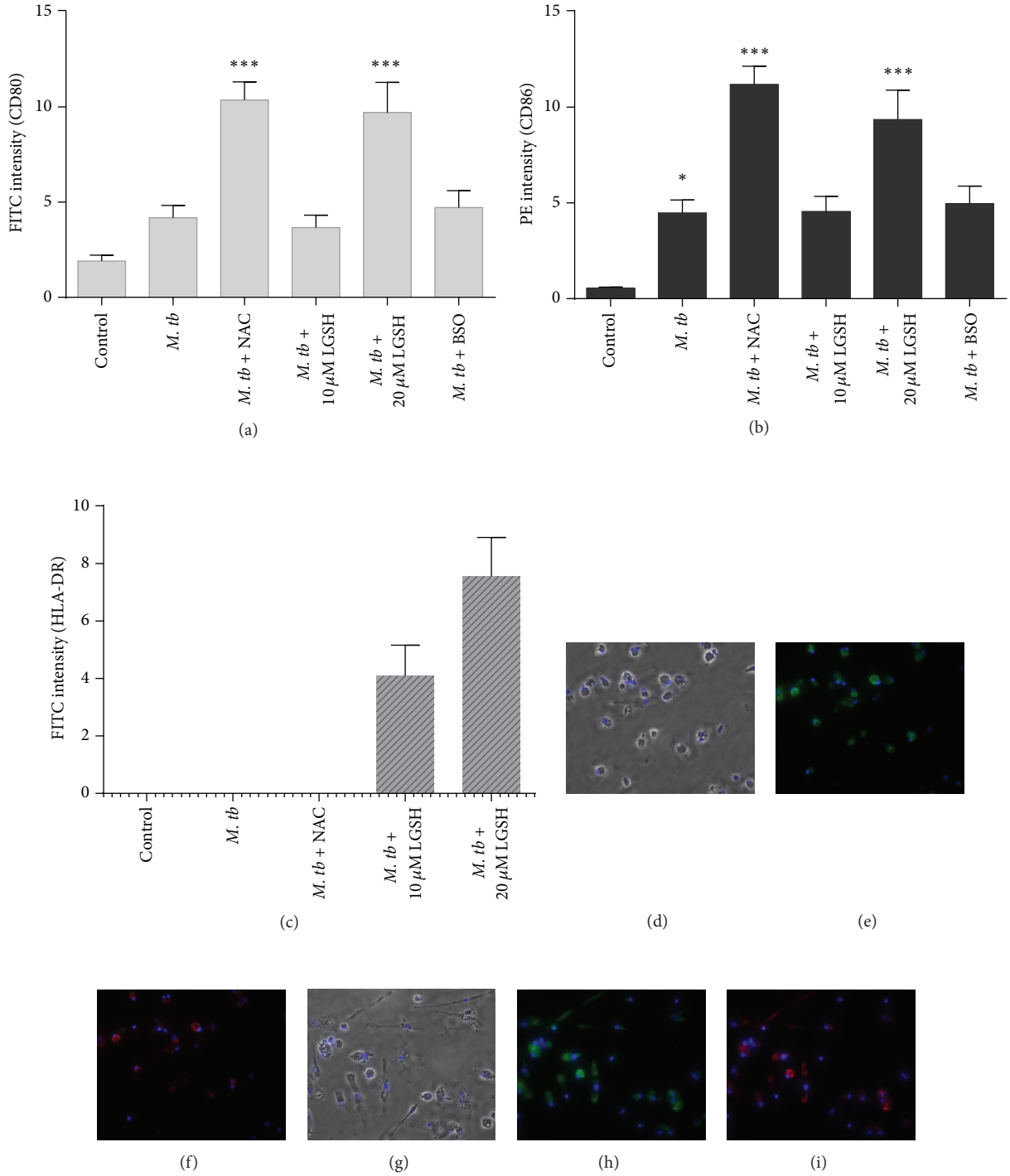

Figure 4: Immunocytochemical analysis of costimulatory marker expressions on the cell surface of DCs. Co-stimulatory marker expression on the surface of DCs was measured by immuno-cytochemistry. Results were analyzed by ANOVA with Dunnett's multiple comparisons test, comparing all treatment categories to the infected-untreated control. Infected-untreated controls were compared to the untreated-uninfected control using the Student's t-test, $^{*}P \leq 0.05$, $^{***}P \leq 0.001$. (a) Quantification of CD80 expression on DCs. (b) Quantification of CD86 expression on DCs. (c) Quantification of HLA-DR expression on DCs. (d)–(i) Fluorescent microscopy images of DCs from control and NAC-treated categories (20x magnification). Antibodies against HLA-DR and CD86 are labeled with FITC 488 (green) and PE 546 (red), respectively. (d) Bright field view of control DCs overlaid with fluorescent microscopy images using DAPI. (e) Control category DCs labeled with FITC conjugated anti- HLA-DR antibodies. (f) Control category DCs labeled with PE conjugated anti-CD-86 antibodies. (g) Bright field view of NAC treated DCs overlaid with fluorescent microscopy images using DAPI. (h) NAC treated DCs labeled with FITC conjugated anti-HLA-DR antibodies. (i) NAC treated DCs labeled with PE conjugated anti-CD-86 antibodies.

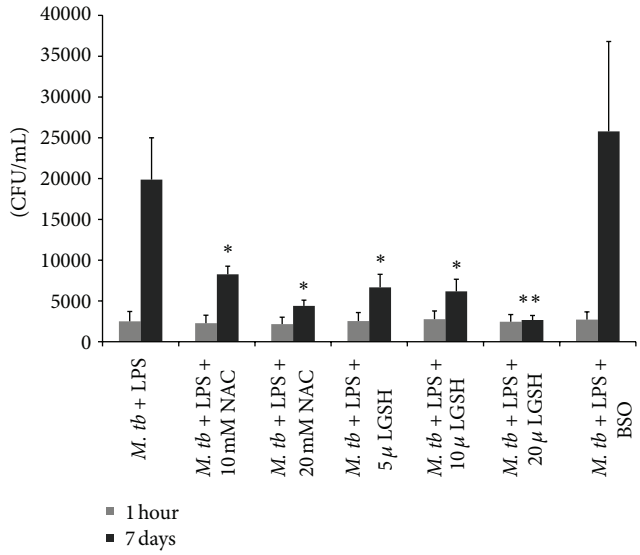

Figure 5: Intracellular survival of H37Rv inside mature DCs. Monocyte derived DCs were infected with processed H37Rv at a multiplicity of infection of 1 : 1 (1 bacterium per DC). Infected DCs were cultured in medium containing LPS (1 μg/mL) in the presence and absence of NAC (5, 10, and 20 mM), L-GSH (5, 10, and 20 μM), or BSO (500 μM). Infected DCs were terminated at 1 hour and 5 days after infection to determine the intracellular survival of H37Rv in mature DCs. Results were analyzed using one-way ANOVA with Dunnett's multiple comparisons test, comparing all treatment categories to the infected-LPS treated control, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

increase in the intracellular growth of H37Rv (Figure 7(b)). In fact, the fold increase in the survival of H37Rv in co-cultures monocytes + NAC-treated Tregs was lowest compared to other treatment groups such as monocytes alone and monocytes + untreated Tregs.

3.11. Assay of IL-10 and TGF-β in Supernatants from H37Rv-Infected Monocytes Cultured in the Presence and Absence of Non-Tregs and Tregs. Results of our studies indicate that Tregs produce the most amounts of immunosuppressive cytokines such as IL-10 and TGF-β (Figures 8(a) and 8(b)) compared to monocytes and non-Tregs. Enhancing the levels of GSH in T cells (Tregs and non-Tregs) resulted in decreased synthesis of both TGF-β and IL-10 (Figures 8(a) and 8(b)) thereby augmenting the host responses to control *M. tb* infection.

4. Discussion

DCs are professional APCs that constitute 0.5%–1% of the leukocyte population in peripheral blood mononuclear cells. Majority of DC populations are present in nonlymphoid tissues and organs including skin, heart, liver, lung, and mucosal surfaces. DCs have the unique ability to link the innate and adaptive immune responses by initiating, stimulating, and regulating T cell responses, including antigen-specific T lymphocytes, Th1/Th2 modulation, Treg induction, and peripheral T cell deletion [12, 13].

DCs can either be myeloid derived DCs or lymphoid-derived DCs [14]. In this study myeloid derived DCs were generated *in vitro* by culturing peripheral blood monocytes in the presence of GM-CSF and IL-4 for 7 days [15].

GSH has been previously reported as an antimycobacterial agent, capable of limiting the intracellular growth of *M. tb* in both murine and human macrophages. Thus, GSH has direct antimycobacterial activity and functions as an effector molecule in innate defense against *M. tb* infection [16–18]. Consistent with these observations, we have also found that GSH in combination with cytokines such as IL-2 and IL-12 enhances the activity of NK cells to control *M. tb* infection inside human monocytes [19]. Importantly, data from our most recent studies indicate that GSH activates the functions of T lymphocytes to control *M. tb* infection inside human monocytes [20]. Finally, we demonstrated that GSH levels are significantly compromised in macrophages, NK cells, and T cells isolated from individuals with HIV infection and this decrease correlated with several-fold increase in the intracellular survival of *M. tb* [5, 18–20]. All these observations support the fact the GSH controls *M. tb* infection by functioning as an antimycobacterial agent as well as by enhancing the functions of NK and T cells, and deficiency of GSH in immune cells derived from individuals with HIV infection is accompanied by diminished control of *M. tb* infection [5, 16–20].

In this study we determined the effects of NAC (5 mM, 10 mM, and 20 mM) and L-GSH (5 μM, 10 μM, and 20 μM) treatments in enhancing the levels of GSH in DCs thereby improving the control of intracellular *M. tb* infection. Although treatment of *M. tb*-infected DCs with NAC (20 mM) or L-GSH (20 μM) resulted in maximum enhancement in the intracellular levels of *r*GSH (Figure 1(c)), we observed maximum inhibition in the growth of H37Rv when DCs were treated with either 10 mM NAC or 5 μM L-GSH (Figure 2). These findings indicate that control of *M. tb* infection is achieved by supplying DCs with delicately balanced optimal concentrations of GSH. Interestingly, maximum inhibition in the growth of H37Rv was observed when DCs were treated with L-GSH at 5 μM concentration (2000-fold lower concentration compared to NAC) (Figure 2). These results are consistent with our previous finding in macrophages, confirming that GSH functions as an effector molecule limiting the intracellular growth of *M. tb* inside DCs [16–18].

We observed that by enhancing the levels of GSH in DCs there is decreased production of IL-1 (Figure 3(a)). IL-1 is a proinflammatory cytokine and excess levels of IL-1 can cause fever, necrosis, and inflammation [5]. It is important to note that the concentrations of NAC/L-GSH that caused maximum reduction in the synthesis of IL-1 are the same concentrations that resulted in most effective inhibition in the intracellular growth of *M. tb* inside DCs (Figure 3(a)).

During intracellular infections, DCs process the antigen, migrate to T cell rich lymph nodes, and present the peptides to T cells via MHC class molecules [21]. IL-12 secretion by DCs will induce naïve T cells to differentiate into Th1 (CD4+)

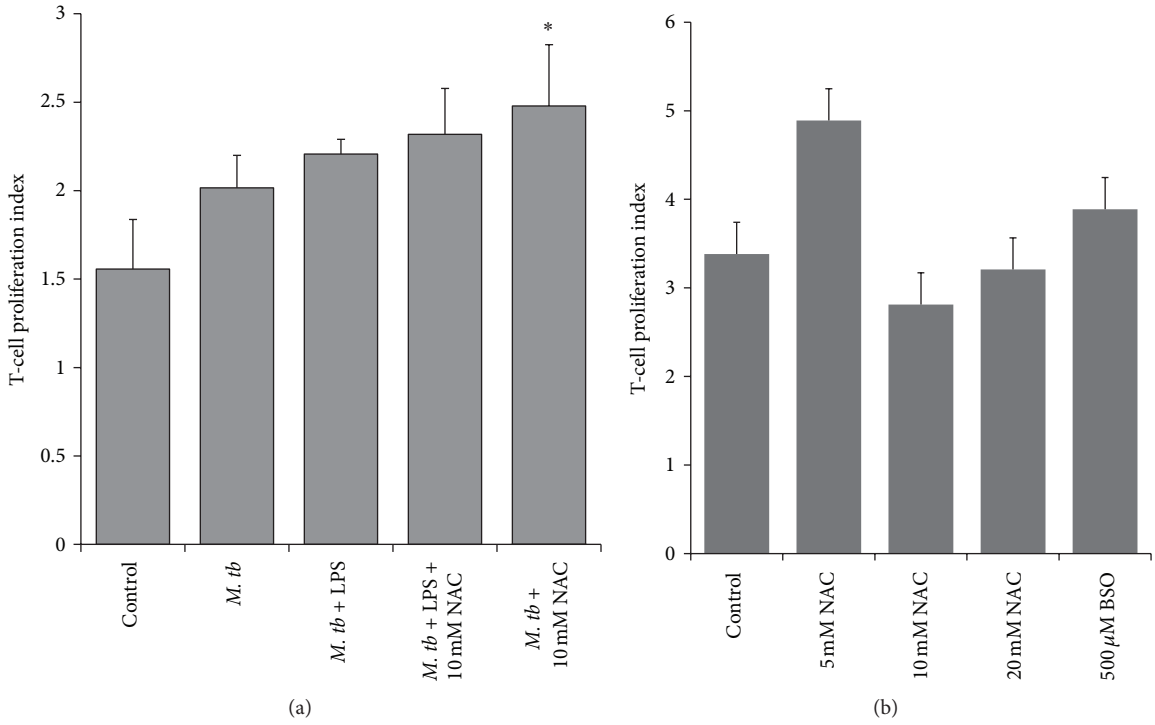

Figure 6: DCs and T cell proliferation assays. (a) Allogeneic T cell proliferation in response to H37Rv-infected, NAC treated DCs. DCs were treated overnight with NAC (10 mM) either alone or in combination with NAC + LPS (1 μg/mL). Following overnight incubation with NAC, DCs were infected with H37Rv, washed, and resuspended in fresh media containing no additives. Allogeneic T cells isolated from PBMCs using a nylon wool column were stained with CFSE (1 μM). Labeled T cells were added to the wells containing infected DCs. Seven days post-incubation, T cells were aspirated and fixed in PFA and analyzed for T cell proliferation using flow cytometry. (b) Proliferation of GSH-enhanced T cells in response to H37Rv-infected DCs. GSH levels in T cells (not DCs) were manipulated by overnight treatment with different concentrations of NAC (5, 10, and 20 mM). Following overnight incubation with NAC, T cells were washed three times with PBS, labeled with CFSE, and then added to the infected DCs to determine the ability of GSH-enhanced T cells to effectively respond to the *M. tb*-infected DCs and proliferate. Seven days after incubation, T cells were aspirated and fixed in PFA and analyzed for T cell proliferation using flow cytometry.

subsets. Th1 cells produce IL-2 and IFN-γ. IFN-γ producing response is crucial for eliminating intracellular pathogens including *M. tb* [22]. Conversely, IL-10 secretion by DCs will induce naïve T cells to differentiate into Th2 subsets. Th2 cells are characterized by the production of IL-4 and IL-5 [22, 23]. These cytokines serve as growth and differentiation factors for B cells, respectively, leading to production of different classes of antibodies. Th2 responses are not considered useful for controlling intracellular infections [23].

In our studies, we observed that increasing the levels of GSH in DCs by treatment with L-GSH (5 μM) induced increased synthesis of IL-12, a cytokine that is responsible for polarizing CD4+ T cells to a Th1 subset (Figure 3(b)). This observation is consistent with the findings of other studies conducted which showed that treatment with lower concentration of NAC promotes IL-12 synthesis [24].

We found that enhancing the levels of GSH in DCs also resulted in decrease in the synthesis of IL-10 (Figure 3(c)). IL-10 is an immunosuppressive cytokine that can polarize the CD4 T cells to Th2 subtype. Therefore, decreased levels of IL-10 will indirectly favor Th1 CD4 T cell response. In contrast, conditions that will result in decreased levels of GSH, such as BSO-treatment, will enhance the synthesis of IL-10 leading to a Th2 CD4+ T cell response (Figure 3(c)). Th2 CD4+ T cell response will promote antibody production and will not result in control of *M. tb* infection and on the contrary will favor the intracellular growth of *M. tb*.

Immature DCs have high phagocytic and endocytic capabilities, and upon stimulation by microbial products or pro-inflammatory cytokines DCs mature into potent APCs.

Upon maturation DCs express activating molecules CD83 or CMRF-44 and co-stimulatory molecules CD40, and B7-family members CD80 and CD86 [25]. DCs also up-regulate their expression of HLA-DR and CD1, MHC class II surface receptors, used to process and present antigens to naïve T cells.

DCs are sentinel cells surveying peripheral tissue as well as lymphoidal tissue for potential pathogens [10]. Upon recognition of a pathogen, DCs phagocytize pathogen and begin their maturation process where they reduce their phagocytic capabilities and begin their migration to the nearest lymph node. In the lymph node they present the pathogen to naïve T-cells via their MHC Class II molecule and co-stimulatory receptors of the B7 family, CD80, 86 and 40 [7].

Mature T cells are characterized by the expression of T cell receptor (TCR) and coreceptors CD4 and/or CD8 [22]. T cells

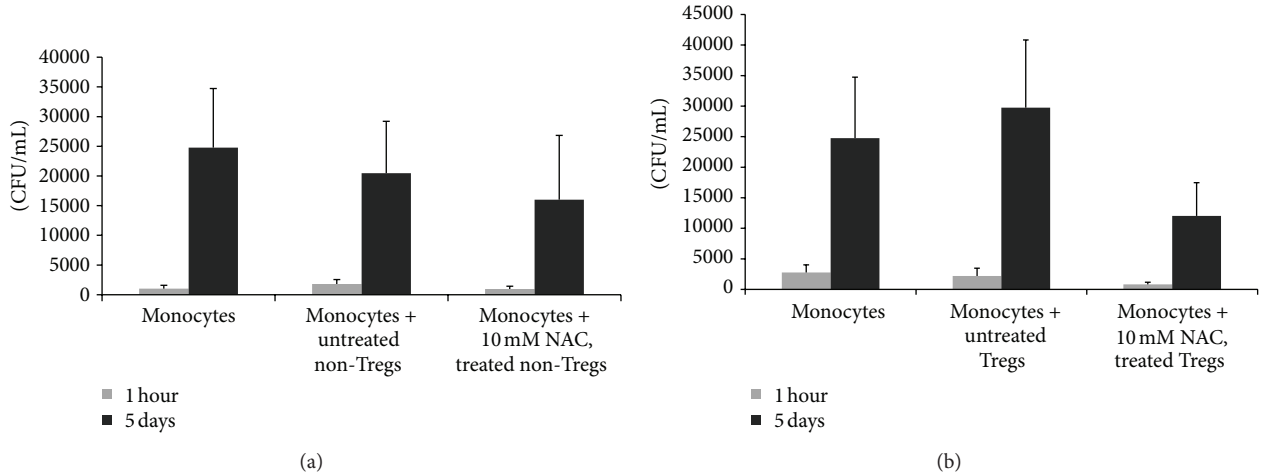

Figure 7: Determination of intracellular viability of H37Rv in cocultures of H37Rv-infected monocytes and CD4+ T cells (non-Tregs and Tregs). Tregs and non-Tregs were isolated from PBMCs derived from healthy subjects using midi-MACS LD columns and mini-MACS MS columns from Miltenyi Biotech. Adherent monocytes were infected with processed H37Rv at a multiplicity of infection of 1 : 1 and incubated for 2 hours for phagocytosis. Unphagocytosed mycobacteria were removed by washing the infected monocytes three times with sterile PBS. Infected monocytes were cultured in RPMI containing 5% AB serum in presence and absence of autologous non-Tregs (a) and Tregs cells (b). Prior to co-incubation with infected monocytes, autologous CD4+ T cells (both Tregs and non-Tregs) were incubated overnight with NAC (10 mM), washed with PBS, re-suspended in fresh RPMI containing AB serum (without any additives), and then added to the infected monocytes (monocyte: T cell ratio was adjusted to 1 : 1). Infected monocyte-CD4+ T cell cocultures were terminated at 1 hour and 5 days after infection to determine the intracellular survival of H37Rv. Infected monocytes cultured in the absence of T cells served as negative controls. Results were analyzed using one-way ANOVA with Dunnett's multiple comparisons test, comparing all T-cell co-culture categories to the monocyte only control. Our results did not achieve statistical significance.

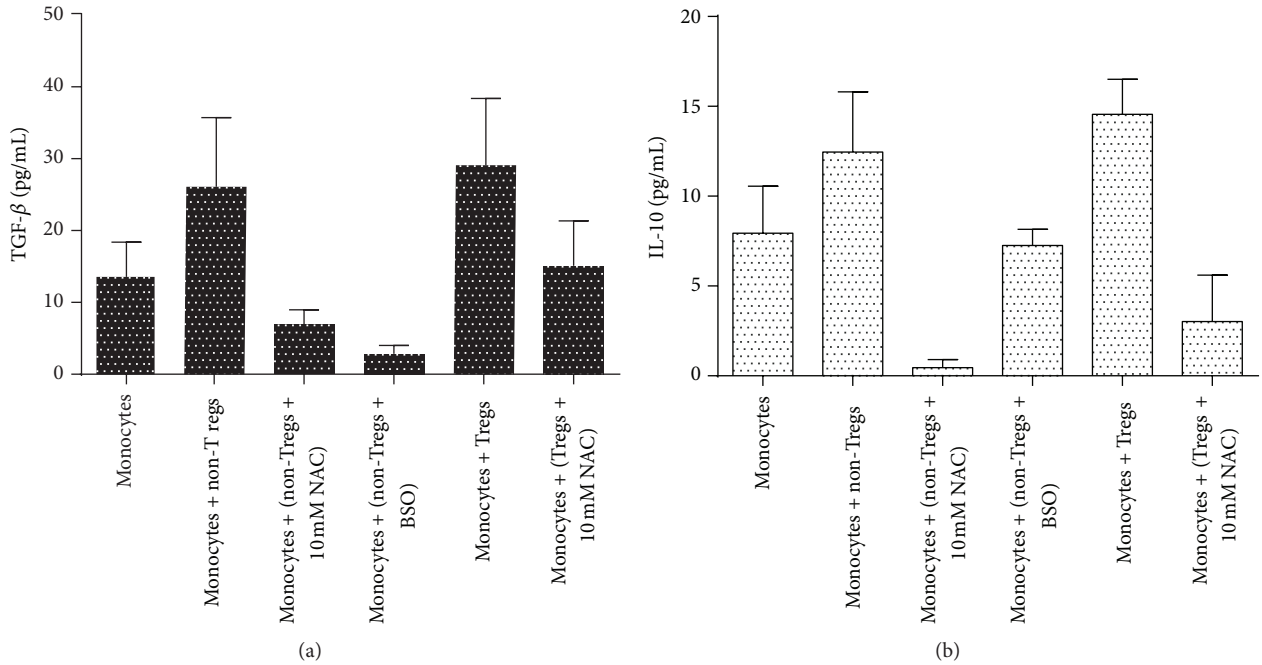

Figure 8: Assay of IL-10 and TGF-β in supernatants from monocytes + CD4+ T-cells. Cytokines in supernatants from monocyte Treg and monocyte-non-Treg co-cultures were measured on $n = 5$ samples by ELISA. Results were analyzed by ANOVA with Dunnett's multiple comparisons test, comparing all treatment categories to the monocyte only control, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. (a) TGF-β. (b) IL-10. Our results did not achieve statistical significance.

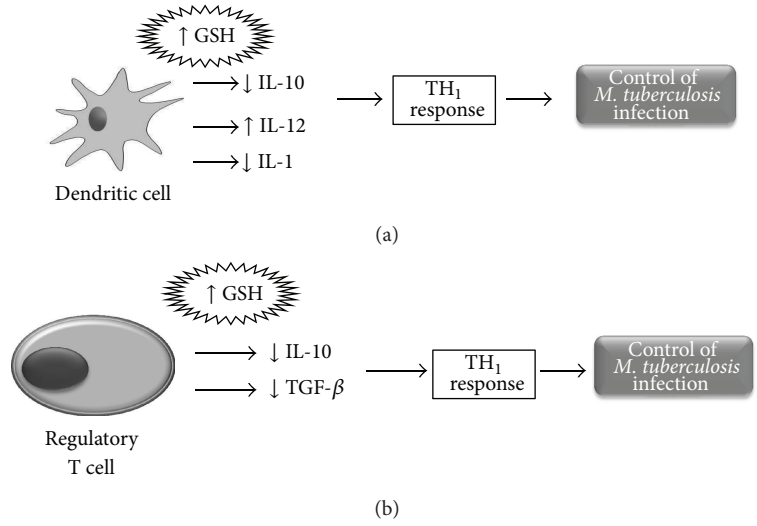

Figure 9: Model describing effects of GSH in growth control of *M. tb*. (a) Enhancing the levels of GSH in DCs decreases the synthesis of IL-10 polarizing the CD4 T cell response to a Th1 type. *M. tb* infection and conditions that will decrease the levels of GSH, such as BSO treatment will enhance the synthesis of IL-10 leading to a Th2 CD4+ T cell response. Th2 CD4+ T cell response will promote antibody production and will not result in control of *M. tb* infection and in contrast will favor the intracellular growth of *M. tb*. Enhancing the levels of GSH in DCs decreases the production of IL-1, a proinflammatory cytokine. Excess of IL-1 can cause fever, necrosis, and inflammation. Enhancing GSH in DCs induces increased synthesis of IL-12 thereby favoring a Th1 CD4+ T cell response. (b) Enhancing the levels of GSH in CD4+ T cells (regulatory and nonregulatory T cells) decreases the synthesis of TGF-β and IL-10 (immunosuppressive cytokines) thereby augmenting the host responses to control *M. tb* infection.

express surface molecules such as CD28 molecule which interacts with DC cell surface molecules B7-1 (CD80) and B7-2 (CD86). Activation of T cells occurs when APCs, such as DCs, present pathogen peptides via major histocompatibility complex I or II (MHC class I or II) to TCR receptors; additionally, a co-stimulatory activation must occur between CD28 molecule and CD80 and CD86 on APCs, the CD28-CTLLA4 interaction [26].

Pathogens such as *M. tb* can inhibit complete maturation and expressions of co-stimulatory molecules that are necessary for migration into lymph nodes by inducing DCs to produce IL-10 [27]. Therefore, the key to *M. tb* control is to enhance macrophage and DC functions.

Results of our studies indicate that in contrast to untreated control DCs (Figures 4(a), 4(b), 4(c), 4(d), 4(e) and 4(f)) treatment with 10 mM NAC and 20 μM L-GSH resulted in upregulation in the expressions of CD80, CD86 and HLA-DR on DCs that were infected *in vitro* with *M. tb* (Figures 4(a), 4(b), 4(c), 4(g), 4(h) and 4(i)).

LPS is a known inducer of DC maturation. Since NAC/L-GSH treatment of DCs not only improved the control of *M. tb* infection by its direct antimycobacterial effects but also upregulated the expressions of HLA-DR, CD80, and CD86, we tested whether treatment of DCs with a combination of NAC/L-GSH + LPS (1 μg/mL) would result in further inhibition in the growth of intracellular *M. tb*. We tested our hypothesis by determining the intracellular survival of H37Rv in NAC/L-GSH + LPS treated DCs. Our results indicate that in comparison to other concentrations of NAC, treatment of DCs with 20 mM NAC in combination with LPS resulted in maximum inhibition in the growth of intracellular *M. tb* (Figure 5). Most importantly, treatment of H37Rv-infected DCs with a combination of LPS + L-GSH (20 μM) resulted in complete stasis in the growth of H37Rv inside DCs (Figure 5). These results indicate that enhancing GSH in DCs in the presence of LPS results in complete stasis in the growth of H37Rv. Notably, L-GSH is able to induce this static effect (in conjunction with LPS) at a 1000-fold lower concentration compared to NAC (Figure 5). Our results indicate that the inhibition in the growth of *M. tb* inside GSH + LPS-treated DCs is due to combination of direct antimycobacterial effects of GSH and oxidative balance. Supplementing DCs with an L-GSH formulation provides complete *r*GSH molecules to cells, circumventing the enzymatic pathway responsible for *r*GSH production, without the requirement for the cell to construct the tripeptide. This may also explain why treatment with L-GSH seems to be more efficacious at much lower concentrations than NAC, as cells treated with NAC will have to produce new molecules of *r*GSH utilizing their own enzymatic machinery.

We also tested the effects of GSH in enhancing the ability of DCs to induce proliferation of allogenic T cells by co-culturing allogeneic T cells with H37Rv-infected DCs. We observed that NAC treatment of DCs induced increased proliferation of allogeneic T cells (Figure 6(a)). Combination of NAC + LPS treatment did not result in further enhancement in the ability of DCs to induce proliferation of allogeneic T cells (Figure 6(a)). These results indicate that NAC treatment alone is sufficient for DCs to induce proliferation of T cells (Figure 6(a)). In separate studies, GSH levels were enhanced in allogeneic T cells (instead of DCs) by treatment with NAC and the effects of GSH supplementation

in enhancing the proliferation of T cells in response to the H37Rv-infected DCs were tested. We observed that treatment of T cells with 5 mM NAC resulted in maximum proliferation when co-cultured with *M. tb*-infected DCs (Figure 6(b)). Our results confirm that GSH enhancement in DCs favor the control of *M. tb* infection. In addition, GSH enhancement upregulates the expressions of co-stimulatory molecules and IL-12, downregulates the synthesis of IL-1 and IL-10, and induces T cell proliferation (Figure 9(a)). However, these changes can occur only at optimum concentration of GSH and the concentration varies for each cytokine and marker, emphasizing the requirement of a fine balance in the levels of GSH to induce favorable changes.

The main groups of T helper cell groups include Th1 cells, Th2 cells, Th17 cells, and Treg cells. Tregs are CD4+CD25+ T-cells which develop and emigrate from the thymus to perform their key role in immune homeostasis through the release of various cytokines [28]. Precise understanding of the immunosuppressive mechanism of Tregs remains elusive, although there is increasing evidence that Tregs manifest their function through various mechanisms that include the secretion of immunosuppressive soluble factors such as IL-10 and TGF-β, cell contact mediated regulation via the high affinity TCR and other co-stimulatory molecules such as CTLA-4, GITR, and cytolytic activity [29, 30]. We tested the intracellular survival of H37Rv inside monocytes co-cultured in the presence and absence of Tregs and non-Tregs. We observed an increased growth of H37Rv inside monocytes that were co-incubated with Tregs (Figure 7(b)). NAC treatment of Tregs resulted in improved control of H37Rv infection inside monocytes (Figure 7(b)). In contrast to monocytes that were co-incubated with Tregs, we observed a reduction in the fold growth of H37Rv when monocytes were cultured in the presence of non-T regs (Figure 7(a)). NAC treatment of non-T regs resulted in further reduction in the fold growth of H37Rv (Figure 7(a)).

Our results also indicate that Tregs produce the highest amounts of immunosuppressive cytokines such as IL-10 and TGF-β (Figures 8(a) and 8(b)) compared to monocytes and non-Tregs. However, when we added NAC we observed a decrease in the amounts of IL-10 and TGF-β production (Figures 8(a) and 8(b)). Our results highlight the role of Tregs in favoring the growth of *M. tb* inside monocytes by producing immunosuppressive cytokines. Furthermore, decrease in IL-10 and TGF-β production following NAC treatment may mitigate the immunosuppressive effects of Tregs cells to allow for an effective immune response (Figure 9(b)) since studies have shown that increased TGF-β and IL-10 can directly suppress the Th1 response which is the first line of defense when battling intracellular infections [31]. Understanding the mechanisms by which Treg cells exert their influence is an area of continued intense research with broad implications for the development of therapeutic strategies for many disease processes including HIV and *M. tb* infection.

Our research indicates that L-GSH and NAC are effective in modulating the levels of pro-inflammatory and immunosuppressive cytokines for beneficial innate and adaptive immune responses against *M. tb* (Figures 9(a) and 9(b)). Our long-term hope is for LGSH/NAC to be a future immune-adjunctive therapy for patients with refractory mycobacterial infections, especially for patients who are immunocompromised.

Conflict of Interests

The authors do not have any conflict of interests. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the paper.

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