Oxidative Medicine and Cellular Longevity

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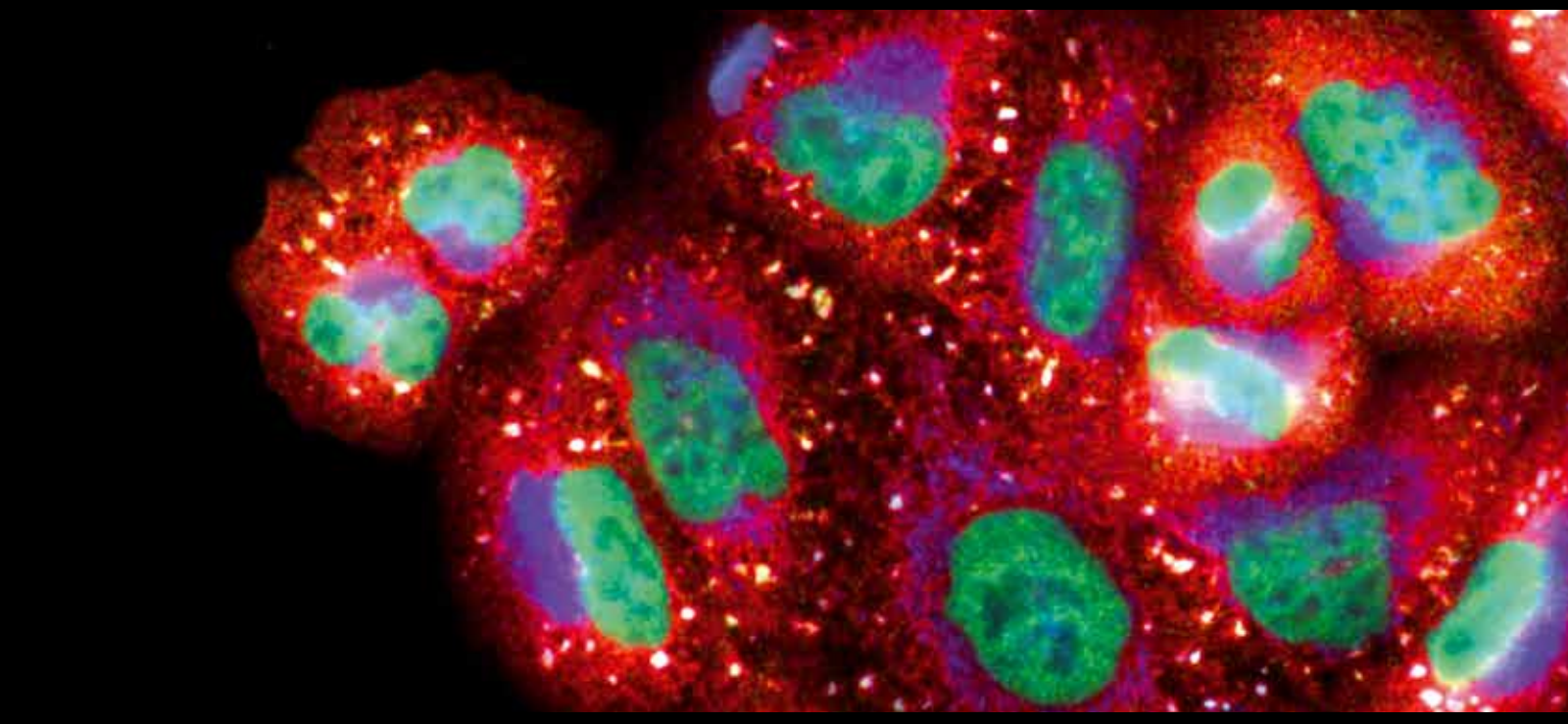
Guest Editors: Darko Modun, Daniela Giustarini, and Dimitrios Tsikas



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Editorial Board

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Editorial

Nitric Oxide-Related Oxidative Stress and Redox Status in Health and Disease

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The molecular oxygen or dioxygen (O_2) molecule has 6 molecular orbitals (MO) in its triplet state, of which the two $\pi_{2p}{}^*$ MO contain each one electron. Thus, O_2 has two unpaired electrons (indicated by a dot $^\bullet$); that is, O_2 is an uncharged diradical molecule: $^\bullet$O–O$^\bullet$. An electron that is donated by another molecule is incorporated in one of the two $\pi_{2p}{}^*$ MO of the O_2 molecule; that is, one $\pi_{2p}{}^*$ MO contains a single unpaired electron. Thus, 1-electron reduction of O_2 yields a negatively charged radical species, that is, the superoxide radical anion ($O_2{}^{-\bullet}$). Subsequent 1-electron reduction of $O_2{}^{-\bullet}$ yields a doubly negatively charged species, that is, the peroxide anion ($O_2{}^{2-}$) which possesses a nonoccupied $\sigma_{2p}{}^*$ MO. Intake of two electrons by the peroxide anion does not form a stable molecule but the bond between the two O atoms breaks to yield two O^{2-} ions which are protonated to form water.

Nitric oxide (NO) is an important signaling molecule with multiple pivotal roles in the cardiovascular and neural systems, as well as in inflammatory response. Due to its unpaired electron, NO is a free uncharged radical, with the unpaired electron being closer to the nitrogen atom of the NO molecule: N$^\bullet$=O. The high affinity of N$^\bullet$=O and $^\bullet$O–O$^\bullet$ to many heme groups-containing proteins, notably hemoglobin, and enzymes, predominantly soluble guanylyl cyclase (sGC), determines both the metabolic fate and the biological activity of NO. Nitric oxide tremendously activates the sGC which catalyses the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). N$^\bullet$=O's high affinity to oxyhemoglobin ($HbFeO_2$) and its rapid Fe-catalysed oxidation to nitrate ($NO_3{}^-$) make direct measurement of NO almost impossible in living organisms (see reaction (R1)) [1]. In heme groups-free aqueous media, authentic NO may exist for several minutes, with nitrite ($NO_2{}^-$) being its major autoxidation product (see reaction (R2)) [1]. Under certain conditions, circulating and excretory nitrite and nitrate are useful indicators and measures of NO synthesis in humans [2]. Many different storage forms of NO have been suggested, with the most important being nitrite and *S*-nitrosothiols (RSNO). In addition to possessing vasodilating and antiplatelet activity, *S*-nitrosothiols can modify protein thiols by *S*-nitrosation and/or *S*-thiolation, thereby altering their inherent activity [3]:

$$\mathrm{HbFe^{(II)}O_2 + NO \longrightarrow HbFe^{(III)} + ONO_2{}^-} \quad \text{(R1)}$$

$$\mathrm{4NO + O_2 + 2H_2O \longrightarrow 2ONO^- + 2ONO^- + 4H^+} \quad \text{(R2)}$$

Oxidative stress, that is, the imbalance between production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) on the one side and antioxidative defense on the other side, is related with numerous diseases, along with some specific conditions such as hyperoxia, ageing, and physical exercise [2]. The direct damaging result of oxidative stress is oxidation of lipids, proteins, and DNA, all of which endanger cell homeostasis. ROS are formed from incomplete reduction of O_2. As mentioned above, intake of one electron (e^-) by one O_2 molecule yields the ROS superoxide radical anion $O_2{}^{-\bullet}$ (see reaction (R3)). The superoxide radical anion itself strongly tends to receive another electron to form the

much more stable peroxide dianion (see reaction (R4)) which is protonated to form hydrogen peroxide (HO–OH, H_2O_2), another ROS.

When $O_2^{-\bullet}$ and $N^{\bullet}$=O meet each other, the unpaired electron of the occupied $\pi_{2p}{}^*$ MO of $N^{\bullet}$=O is transferred to the singly occupied $\pi_{2p}{}^*$ MO of $O_2^{-\bullet}$; that is, the N atom is oxidized from the oxidation state +2 to the oxidation state +3, while the O atoms of $O_2^{-\bullet}$ are reduced (from the oxidation state −1/2 to −1). This extremely rapid reaction (see reaction (R5)) yields the RNS peroxynitrite (O=N–O–O^-). Because the two O atoms of the peroxide group of peroxynitrite are incompletely reduced and the N atom of peroxynitrite is incompletely oxidized, peroxynitrite is highly reactive at physiological pH values. Intramolecular transfer of two electrons from the N atom to the peroxide group and its rearrangement yields nitrate (see reaction (R6)). In the presence of other biomolecules such as reduced glutathione (GSH; see reaction (R7)) or tyrosine (TyrH; see reaction (R8)) the two missing electrons are provided by the biomolecules which themselves are oxidized, for instance, GSH to glutathione disulfide (GSSG) and TyrH to 3-nitrotyrosine:

$$^{\bullet}O–^{\bullet}O + \mathbf{e}^- \longleftrightarrow {}^{\bullet}O–O^- \quad (R3)$$

$$^{\bullet}O–O^- + \mathbf{e}^- \longleftrightarrow {}^{-}O–O^- \quad (R4)$$

$$^{-}O–O^{\bullet} + N^{\bullet}=\mathbf{O} \longleftrightarrow {}^{-}O–O–N = \mathbf{O} \quad (R5)$$

$$^{-}O–O–N=\mathbf{O} \longrightarrow O(\mathbf{O})N\mathbf{O}^- \quad (R6)$$

$$2\ {}^{-}O–O–N=\mathbf{O} + 2GSH \longrightarrow 2\ {}^{-}O–N=\mathbf{O} + GSSG + H_2O_2 \quad (R7)$$

$$^{-}O–\mathbf{O}–N=\mathbf{O} + TyrH \longrightarrow \mathbf{O}–(\mathbf{O}=)N–Tyr + HO^- \quad (R8)$$

Modifications of biomolecules induced by ROS and/or RNS may alter the physiological function of the biomolecules and may have severe consequences for the organism [4]. In addition, oxidative stress may also reduce nitric oxide's bioavailability, because oxidative reaction products of NO including nitrite and peroxynitrite are only very ineffective sources for NO. Furthermore, oxidative stress may cause uncoupling of the endothelial nitric oxide synthase (eNOS), thus diminishing NO synthesis/bioavailability and aggravating NO-dependent oxidative stress. Excessive, uncontrolled, and unmanaged oxidative stress is certainly hazardous to humans and leads to diseases. Therefore, the prooxidative and antioxidative state in cells and tissues determines decisively synthesis and bioactivity not only of NO, but also of *S*-nitrosothiols and nitrite in health and disease, as well as in different conditions such as physical exercise and smoking.

In consideration of the eminent importance of NO-related oxidative stress and redox status in health and disease, we organized the present special issue. We are very pleased to present to the readership of the journal and to the general scientific community interested in this topic this special issue.

This thematic volume includes review and research articles. In their review paper, M. A. Abdelmegeed and B.-J. Song describe approaches for identifying nitrated proteins and studying their roles in promoting liver diseases and discuss translational research applications. S. Savvanis et al. report that sildenafil (Viagra), an inhibitor of the phosphodiesterase (PDE) isoform 5, has beneficial effects in a rat model of liver ischemia/reperfusion which is associated with elevated oxidative stress. Obstructive sleep apnea (OSA) is considered an independent risk factor for cerebrovascular and cardiovascular diseases. Paradoxically, hypoxia, which is a major pathophysiological feature of OSA, can enhance oxidative stress. In their review article, M. Badran et al. discuss the role of oxidative stress in OSA and its causal effect on endothelial dysfunction by interaction with NO in animal models and in humans. In patients with microvascular angina, B. Porro et al. investigated the interrelationship of NO and oxidative stress. Their study indicates that altered microvascular bed is associated with impaired capacity of red blood cells to produce NO, presumably due to elevated oxidative stress on the basis of the erythrocytic GSSG/GSH molar ratio as measured by LC-MS/MS.

With respect to the L-arginine/nitric oxide, children are not small adults [5]. N. K. Kanzelmeyer et al. quantified the L-arginine/nitric oxide in children with haemolytic-uraemic syndrome (HUS) and healthy controls by GC-MS. An interesting finding of this study was the close positive correlation between plasma nitrate and plasma free haemoglobin concentration. The authors discuss that elevated free haemoglobin in plasma of HUS children can not only oxidize NO to nitrate outside of the erythrocytes, but also increase oxidative stress. K. Pimková et al. quantified circulating aminothiols, nitrite, nitrate, and malondialdehyde, a widely used biomarker of oxidative stress, in patients suffering from myelodysplastic syndromes (MDS) in the context of clinical outcomes and as a consequence of iron overload. The authors found that oxidative stress is elevated in MDS, presumably independent of iron overload. These findings may bring new insight into the problematic nature both of MDS and oxidative stress.

C. M. O. Volpe et al. found that the production of NO, IL-6, and TNFα in cultured palmitate-stimulated PBMNCs or in plasma from type 2 diabetes mellitus patients or nondiabetic controls is elevated due to hyperglycaemia which is generally associated with elevated oxidative stress. R. Carnevale et al. explored an ex vivo experimental model in humans, in which blood cells are activated to produce ROS via NOX2, namely, the catalytic subunit of NADPH oxidase. The Steen solution, a physiological human serum albumin- and dextran-containing salt solution that is especially used in lung transplantation, was found to possess antioxidant properties via downregulation of NADPH oxidase activity and to enhance NO production. *N*-[3-(Aminomethyl)benzyl]acetamidine (1400 W) is considered a highly selective inhibitor of inducible nitric oxide synthase (iNOS). A. Mertas et al. investigated the effect of 1400 W on NO, IL-12, and TNFα production by LPS and TNFγ activated J774A.1 macrophages. The authors concluded that the potency and selectivity of 1400 W as an inhibitor of iNOS and cytokine release modifier are encouraging for therapeutic use of 1400 W.

Paracetamol (acetaminophen) is a widely used analgesic and antipyretic drug. Paracetamol readily reacts with RNS including peroxynitrite to form 3-nitro-paracetamol and di-paracetamol, in analogy to tyrosine. D. Tsikas et al. demonstrated by LC-MS/MS and GC-MS/MS the appearance of 3-nitro-paracetamol and di-paracetamol in plasma and urine samples of healthy subjects who received orally a single 500 mg paracetamol tablet and suggested a novel human model of oxidative stress based on oral paracetamol administration [6]. Application of such a model of oxidative stress to humans requires that paracetamol does not alter oxidative stress. Therefore, this group investigated the effects of paracetamol in vitro and in vivo studies in humans on the activity of enzymes that are known to produce superoxide. A. Trettin et al. found that paracetamol does not change oxidative stress, even not at the suprapharmacological single oral dose of 3 g in healthy male subjects. The utility of the paracetamol model to measure oxidative stress in health and disease remains to be demonstrated.

In summary, the present special issue assembles information from studies performed in cell systems in vitro, in animals and in human studies. The articles collected in the special issue address current standing and progress in the area of oxidative stress in relation to NO, its implication in disease, and the effect of antioxidants and drugs on NO-related dysfunction. Included articles also address models and mechanisms of NO-related oxidative stress in vitro, in animals and in humans, and provide new ideas and concepts to better understand the complex and challenging nature of oxidative stress. Delineating mechanisms are essential to effectively prevent oxidative stress and to specifically improve intra- and extracellular redox status, that is, where really required, without affecting pathways nonrelated to NO.

Before closing the editorial, we would like to express our sincere thanks to the authors for their valuable contributions and to the reviewers who helped a lot to improve the quality of the articles published herein. We hope that this work will be of help in the exciting and challenging area of NO-related oxidative stress.

Darko Modun
Daniela Giustarini
Dimitrios Tsikas

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Research Article

N-[3-(Aminomethyl)benzyl]acetamidine (1400 W) as a Potential Immunomodulatory Agent

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This study was designed to investigate the relationship between NO, IL-12, and TNF-α production by J774A.1 macrophages activated with LPS and IFN-γ in the presence of N-[3-(aminomethyl)benzyl]acetamidine (1400 W). 1400 W is a novel, highly selective inhibitor of inducible nitric oxide synthase (iNOS). We compared the obtained data with the effect of N^G-monomethyl-L-arginine (L-NMMA) (a nonselective NOS inhibitor) and L-N^G-(1-iminoethyl)lysine (L-NIL) (a relatively selective inhibitor of iNOS activity) on cells in this model. To investigate the involvement of an exogenous NO on IL-12 and TNF-α production we used NO donor—S-nitrosocaptopril (S-NO-Cap). The most potent inhibitor of NO generation was 1400 W. This compound also markedly increased IL-12 p40 secretion and decreased TNF-α release. L-NIL suppressed both NO and TNF-α production, but it did not change IL-12 p40 synthesis. The effect of L-NMMA on NO generation was weaker than other inhibitors. Moreover, it decreased TNF-α secretion slightly but not significantly. IL-12 p40 production by stimulated cells was inhibited by S-NO-Cap in a dose dependent manner, but no effect on TNF-α release was observed. The potency and selectivity of 1400 W as an inhibitor of iNOS and cytokine release modifier are encouraging for therapeutic use.

1. Introduction

Cytokines are low molecular weight polypeptides that initiate the inflammatory response and define the magnitude and the nature of the acquired immune response. Interleukin 12 (IL-12), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) are three inflammatory mediators, which have significant impact on the cytokine balance and type of immune response. Additionally, nitric oxide (NO) seems to participate in this regulation [1, 2]. IL-12 (a heterodimer composed of two subunits: p35 and p40) induces commitment from the T helper 0 (Th0) to Th1 phenotype [3, 4]. NO has been suggested to inhibit IL-12 transcription and to act as negative feedback on Th1 cell development [5]. Also TNF-α seems to be a specific inhibitor of IL-12 p40 secretion from human macrophages [6].

IL-12, TNF-α, and NO are produced and released by macrophages upon activation by a variety of immunological stimuli, such as lipopolysaccharide (LPS) and cytokines. NO is synthesized from L-arginine by the enzyme—NO synthase (NOS), which is either constitutive (endothelial—eNOS and neuronal—nNOS) or induced (iNOS) by bacterial products and cytokines [7, 8]. High output NO production from activated macrophages is a result of iNOS expression [9, 10]. There are known several NOS inhibitors; most of them are analogs of the substrate L-arginine [11]. Preservation of physiologically important NOS functions might require use of isoform-selective inhibitors. N-[3-(aminomethyl)benzyl]acetamidine (1400 W) is a slow, tight binding, and highly selective inhibitor of iNOS [12, 13].

The purpose of this study was to investigate the relationship between NO, IL-12, and TNF-α production by J774A.1 macrophages activated with LPS and IFN-γ in the presence of 1400 W. We compared obtained data with the effect of N^G-monomethyl-L-arginine (L-NMMA) (a nonselective NOS inhibitor) and L-N^G-(1-iminoethyl)lysine (L-NIL) (a

relatively selective inhibitor of iNOS activity) on cells in this model. To investigate the involvement of an exogenous NO on IL-12 and TNF-α production we used NO donor—S-nitrosocaptopril (S-NO-Cap).

The J774A.1 cell line was used in our study because this kind of cells is a widely used useful model to study the process of nitric oxide (NO) synthesis. In J774A.1 murine monocyte-macrophage cell line NO production significantly increases in the presence of LPS and IFN-γ, or LPS alone [14–17].

2. Materials and Methods

2.1. Reagents. 1400 W, L-NIL, L-NMMA, and S-NO-Cap were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). LPS from *Escherichia coli* serotype O127:B8 and trypan blue were purchased from Sigma Chemical Company (St. Louis, MO, USA). Recombinant mouse IFN-γ was obtained from Genzyme Corporation (Cambridge, MA, USA).

2.2. Cell Culture. The mouse macrophage cell line J774A.1 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were maintained in an atmosphere of 5% CO_2, at 37°C in RPMI 1640 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL Life Technologies, Paisley, UK). The cells were cultured in 50 cm^2 plastic flasks (Nunc A/S, Roskilde, Denmark). For experiments cells were detached by vigorous pipetting and, after centrifugation, suspended in fresh medium. Macrophages at a density of 1×10^6 cells/mL were activated with a combination of LPS (100 ng/mL) and IFN-γ (25 U/mL) for 18 h. Incubations were performed in 24-well plates (Nunc A/S, Roskilde, Denmark) in the presence or absence of iNOS inhibitors or S-NO-Cap.

2.3. NO Generation by Stimulated J774A.1 Macrophages. Nitrite concentrations as a stable final product of NO were measured by a colorimetric Griess method as described previously [18]. Briefly, equal volumes of cell culture supernatants and Griess reagent (0.5% sulfanilamide, 0.05% naphtylene-diamide dihydrochloride in 2.5% H_3PO_4) were mixed and incubated in room temperature for 10 min. The absorbance values were determined at 550 nm with an automated microplate reader Elx800 (BIO-TEK Instruments Inc., Winooski, VT, USA). As a standard, sodium nitrite was used. Data were expressed as μM nitrite per 10^6 cells originally plated.

2.4. IL-12 p40 Production. The concentration of studied cytokine in culture cell supernatants was quantitated using a sandwich ELISA. The Mouse IL-12 p40 Immunoassay Kit was purchased from R&D Systems (Minneapolis, MN, USA). ELISA was developed with horseradish peroxidase-conjugated antibody against mouse IL-12 p40 followed by tetramethylbenzidine substrate. The absorbance was read on a microplate reader Elx800 (BIO-TEK Instruments Inc., Winooski, VT, USA) at 450 nm. Recombinant murine IL-12 was used as a standard. This assay has a sensitivity of detection <4 pg/mL.

2.5. TNF-α Production. Immunoreactive TNF-α was estimated in cell culture supernatants by a double-antibody ELISA kit using recombinant murine TNF-α as a standard (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. The absorbance values were measured at 450 nm using the microplate reader Elx800 (BIO-TEK Instruments Inc., Winooski, VT, USA). The sensitivity of the assay was <5.1 pg/mL.

2.6. Determination of Cell Viability. Cell viability was determined by trypan blue dye exclusion and was assessed biochemically by measuring the cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) using Cytotoxicity Detection Kit (Boehringer Mannheim, Mannheim, Germany). LDH activity in cell culture supernatants was measured as the amount of pyruvate consumed because of oxidation of NADH. The absorbance values were determined at 490 nm using a microplate reader Elx800 (BIO-TEK Instruments Inc., Winooski, VT, USA).

2.7. The Statistical Analysis. In our study each experiment was performed in quadruplicate, as the two independent experiments performed in duplicate ($n = 4$ in each group). The results are presented as the arithmetic mean and the median. The statistical differences between groups were determined by analysis of variance followed by the unpaired Student's t-test and the Mann-Whitney U test, depending on how well the results correlated with a normal distribution. Differences between the mean values were considered to be statistically significant at $P < 0.05$. The STATISTICA version 10 software (StatSoft, Cracow, Poland) was used to perform the statistical analysis.

3. Results

The viability of the cells was greater than 92% in all performed experiments as determined by a trypan blue staining (data not shown) and LDH release (Figure 1). The accumulated nitrite in cell culture supernatants was used to estimate NO generation. The nitrite, IL-12 p40, and TNF-α levels were determined after 18 h of stimulation with LPS (100 ng/mL) and IFN-γ (25 U/mL) in the presence or absence (control) of iNOS inhibitors.

Unstimulated macrophages released a small but detectable amount of nitrite (0.82 ± 0.58 μM), IL-12 p40 (171.5 ± 24.8 pg/mL), and TNF-α (120.5 ± 2.1 pg/mL) during 18 h incubation (data not shown). Upon activation cells produced 28.9 ± 1.7 μM of nitrite, 2569 ± 393 pg/mL of IL-12 p40, and 1585 ± 358 pg/mL of TNF-α.

In the next experiment S-NO-Cap was investigated for its involvement in cytokines secretion (Figure 2). NO generated by 200 μM of S-NO-Cap significantly increased IL-12 p40 and TNF-α production by unstimulated cells ($P < 0.05$ and $P < 0.001$, resp.). A lower dose of S-NO-Cap (50 μM)

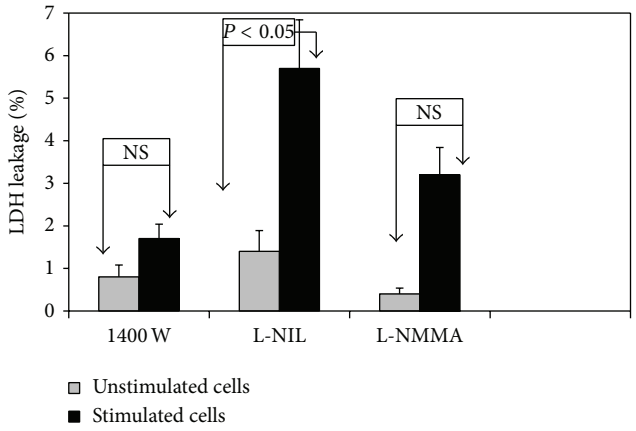


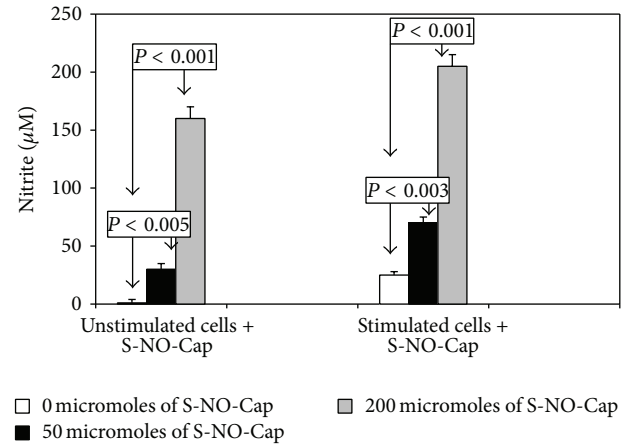
Figure 1: Effect of nitric oxide synthase (NOS) inhibitors (1400 W, L-NIL, and L-NMMA) on LDH release by J774A.1 macrophages. Duplicate cultures of cells (10^6 cells/mL) were incubated at 37°C in the presence or absence (control) of stimuli: LPS (100 ng/mL) and IFN-γ (25 U/mL) for 18 h. Then cell culture supernatants were harvested and examined as described in Materials and Methods. All values represent means ± SD of two independent experiments performed in duplicate ($n = 4$).

affected only TNF-α release ($P < 0.05$). As expected, IL-12 p40 production by stimulated cells was strongly inhibited by S-NO-Cap, but no effect on TNF-α release was observed.

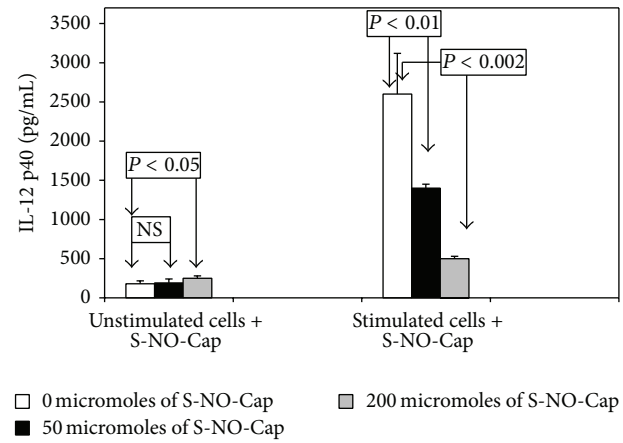
The effect of iNOS inhibitors on NO, IL-12 p40, and TNF-α production by activated J774A.1 macrophages is shown in Figure 3. Cells were stimulated with LPS (100 ng/mL) and IFN-γ (25 U/mL) for 18 h in the presence or absence (control) of 1400 W (50 μM), L-NIL (50 μM), or L-NMMA (100 μM). The most potent inhibitor of NO generation was 1400 W (7.5 ± 0.7% of control, $P < 0.002$). This compound also markedly increased IL-12 p40 secretion (163.8 ± 12.1% of control, $P < 0.001$) and decreased TNF-α release (44.8 ± 0.7% of control, $P < 0.02$). L-NIL suppressed both NO and TNF-α production, but it did not change IL-12 p40 synthesis. The effect of L-NMMA on NO generation was weaker than other inhibitors. Moreover, it decreased TNF-α secretion slightly (86.8 ± 5.9% of control) but not significantly.

4. Discussion

4.1. Effect of an Exogenous NO. In the present study an exogenous NO (generated by S-NO-Cap) affected cytokine release by macrophages. It markedly suppressed IL-12 production by LPS/IFN-γ induced J774A.1 macrophages, which is in agreement with Huang et al.'s report [5], in which NO generated by S-nitroso-N-acetyl-penicillamine inhibits the production of IL-12 protein and IL-12 p40 mRNA expression. NO did not change TNF-α synthesis in our model of stimulated cells. Earlier it was reported that NO might both enhance [19] and attenuate TNF-α production [20] in the different cell lines. Moreover, we showed that treatment of unstimulated J774A.1 cells with NO donor resulted in the increase of the basal levels of IL-12 and TNF-α. NO could control the Th1 cell development through a feedback mechanism that suppressed



(a)



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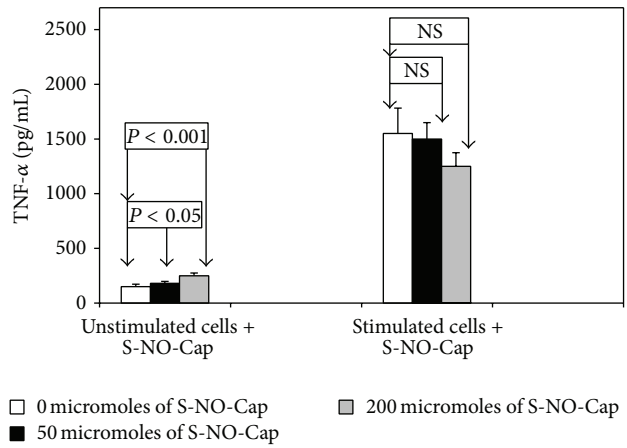

(c)

Figure 2: Effect of S-nitrosocaptopril (S-NO-Cap) on nitrite (a), IL-12 p40 (b), and TNF-α (c) production by activated J774A.1 macrophages. Duplicate cultures of cells (10^6 cells/mL) were stimulated at 37°C with LPS (100 ng/mL) and IFN-γ (25 U/mL) in the presence (50 or 200 μM) or absence (control) of S-NO-Cap for 18 h. Then cell culture supernatants were harvested and examined as described in Materials and Methods. All values represent means ± SD of two independent experiments performed in duplicate ($n = 4$).

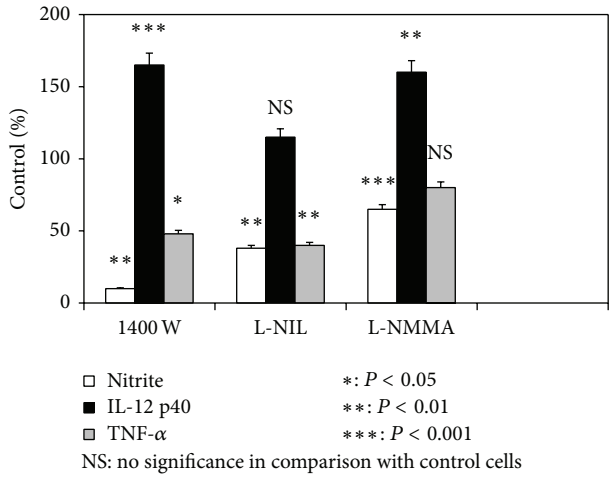

Figure 3: Effect of nitric oxide synthase (NOS) inhibitors on nitrite, IL-12 p40, and TNF-α production by activated J774A.1 macrophages. Duplicate cultures of cells (10^6 cells/mL) were stimulated at 37°C with LPS (100 ng/mL) and IFN-γ (25 U/mL) in the presence or absence (control) of tested compounds: 50 μM of N-[3-(aminomethyl)benzyl]acetamidine (1400 W), 50 μM of L-N^G-(1-iminoethyl)lysine (L-NIL), and 100 μM of N^G-monomethyl-L-arginine (L-NMMA). After 18 h cell culture supernatants were harvested and examined as described in Materials and Methods. Control cells produced 28.9 ± 1.7 μM of nitrite, 2569 ± 393 pg/mL of IL-12 p40, and 1585 ± 358 pg/mL of TNF-α. All values represent means ± SD of two independent experiments performed in duplicate ($n = 4$).

IL-12 synthesis [5]. Taken together, our results confirmed the regulatory effect of NO on cytokine release.

4.2. Effect of NOS Inhibitors. Overexpression of individual NOS isoforms plays a role in a wide range of disorders, including septic shock, arthritis, asthma, diabetes, ischemia-reperfusion injury, and the various neurodegenerative diseases [8, 9, 21]. Knowledge of the physical locations and functions of the various isoforms of NOS is necessary when considering manipulations of their expression. Unfortunately, nonselective NOS inhibitors also inactivate the constitutive isoforms and their administration might cause a marked and sustained increase in blood pressure.

NO production by J774A.1 macrophages has been mainly attributed to inducible NO synthase (iNOS) activity, which is induced by inflammatory cytokines or bacterial products, such as LPS. The iNOS has become a reliable biomarker for fully activated macrophages, whereas information regarding roles for endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase during macrophage activation has been limited [22].

1400 W, a nontoxic for the cells novel NOS inhibitor, is the most selective inhibitor of iNOS isoform described to date [11, 12, 23]. It is greater than 5000-fold and 200-fold more potent against purified human iNOS than eNOS and nNOS, respectively [12]. Treatment with 1400 W at effective doses on iNOS did not have effect on basal systemic blood pressure or on exacerbating early effect of LPS on vascular leakage, whereas treatment with nonselective inhibitor N-nitro-L-arginine methyl ester (L-NAME) caused a significant increase in blood pressure and exacerbation of early vascular leak [24]. A beneficial action of 1400 W on the colonic injury using an experimental model of colitis in rats has been reported [25, 26]. Treatment with 1400 W reduced neutrophil infiltration, edema formation, and acute inflammatory damage in induced acute colitis [25]. It was a potent inhibitor (150-fold more potent than L-NMMA) of LPS-provoked colonic vascular injury in rat model [12, 24]. L-NIL, a relatively selective inhibitor of iNOS, suppressed the increase in the plasma nitrite levels and joint inflammation associated with adjuvant-induced arthritis. Inhibition was observed at doses, which did not appear to inhibit eNOS, as determined by a lack of effect on systemic blood pressure [27]. L-NIL was considerably more potent than L-NMMA in suppressing nitrite accumulation by intact macrophages [28]. Ruetten et al. suggest that selective inhibitors of iNOS activity might attenuate the liver and pancreatic dysfunction caused by endotoxemia in rats [29].

Considerable evidences suggest that 1400 W exerts an anti-inflammatory activity and it is related to its ability of suppressing NO generation. Additionally, this inhibitor could modify the host immune response via the affectation other mediators production. Indeed, we showed that 1400 W decreased TNF-α release. TNF-α, a principle proinflammatory cytokine, is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis. It produces fever, inflammation, tissue destruction, and (in some cases) shock and death [30]. Moreover, 1400 W potently increased a secretion of p40 subunit of IL-12. Interestingly, a p40 homodimer may function as an IL-12 antagonist by binding to the IL-12 receptor, but not by mediating a biologic response [31]. The potency of inhibition of NO production is 1400 W > L-NIL > L-NMMA, but neither IL-12 p40 nor TNF-α production follows this pattern. It is possible because IL-12 production could be perturbed by the excess of endogenous NO. For example, Suzuki et al. [13] suggest that IL-12 production in stimulated J774A.1 cells inhibited by 1400 W could be increased by limiting endogenous NO production.

The potency and selectivity of 1400 W as an inhibitor of iNOS and cytokine release modifier are encouraging for therapeutic use. However, the designing of appropriate strategies for an intervention requires further studies *in vitro* and *in vivo*.

Conflict of Interests

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

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Research Article

Sildenafil Attenuates Hepatocellular Injury after Liver Ischemia Reperfusion in Rats: A Preliminary Study

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We evaluated the role of sildenafil in a rat liver ischemia-reperfusion model. Forty male rats were randomly allocated in four groups. The sham group underwent midline laparotomy only. In the sildenafil group, sildenafil was administered intraperitoneally 60 minutes before sham laparotomy. In the ischemia-reperfusion (I/R) group, rats were subjected to 45 minutes of hepatic ischemia followed by 120 minutes of reperfusion, while in the sild+I/R group rats were subjected to a similar pattern of I/R after the administration of sildenafil, 60 minutes before ischemia. Two hours after reperfusion, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured and histopathological examination of the lobes subjected to ischemia as well as TUNEL staining for apoptotic bodies was performed. Additionally, myeloperoxidase (MPO) activity and the expression of intercellular adhesion molecule-1 (ICAM-1) were analyzed. Serum markers of hepatocellular injury were significantly lower in the sild+I/R group, which also exhibited lower severity of histopathological lesions and fewer apoptotic bodies, as compared to the I/R group. The I/R group showed significantly higher MPO activity and higher expression of ICAM-1, as compared to the sild+I/R group. Use of sildenafil as a preconditioning agent in a rat model of liver I/R exerted a protective effect.

1. Introduction

Liver resections under some type of vascular control are currently favored by many surgeons since they can ensure a less hemorrhagic surgical field by taking advantage of liver tolerance to normothermic warm ischemia [1, 2]. Although such maneuvers are invaluable in preventing excessive blood loss and allow the performance of a safer procedure, they are invariably complicated by ischemia/reperfusion (I/R) injury [3]. Moreover, hepatic I/R injury can also occur in various other clinical contexts, including liver transplantation, hypovolemic shock, and low-output syndrome [4, 5]. In particular, ischemia leads to depletion of cellular energy, accumulation of intracellular sodium, calcium, and reactive oxygen species (ROS), and activation of multiple enzyme systems leading to cell damage [3]. The reestablishment of blood flow through reperfusion can aggravate local tissue injury secondary to an ensuing acute inflammatory response. Reperfused tissue is infiltrated by activated polymorphonuclear leukocytes and platelets while further tissue damage is mediated through cytokine production by leukocytes, complement activation, local imbalance in nitric oxide (NO) levels, accumulation of platelet activating factors and endothelial-cell adhesion molecules, and finally formation of free radicals [6, 7]. This

overwhelming inflammatory response manifests as vasoconstriction, intravascular hemoconcentration, neutrophil migration and adherence, and platelet aggregation [8–10]. The ensuing microcirculatory failure can finally culminate in hepatocellular apoptosis and necrosis with repercussions for the liver as well as distant organs [11–13].

NO is a key molecule, which is recognized as an important, yet controversial mediator of physiological and pathological processes inherent in I/R injury since it has been shown to have both protective and deleterious effects on cellular functions [14]. It has been shown to act through a variety of second-messenger cascades although the majority of its effects are mediated through cyclic guanosine monophosphate (cGMP), which is in turn catabolised by phosphodiesterase type 5 (PDE5) that converts cGMP into the inactive GMP and terminates its action [15]. NO is synthesized from L-arginine by three isoforms of the NO synthase (NOS), the endothelial synthase (eNOS), the inducible synthase (iNOS), and the neuronal synthase (nNOS) [16]. eNOS is responsible for the production of basal NO, which maintains normal vascular tone. iNOS, contrary to eNOS, is calcium insensitive and is especially induced under oxidative stress conditions, with controversial results regarding its role in ischemia-reperfusion [16, 17]. Neuronal (nNOS) is involved in neural signaling with no participation in the ischemia-reperfusion events [18].

eNOS-derived NO is considered to have a cytoprotective effect in I/R injury with cGMP playing an important role in regulation of intracellular calcium levels and favorable modulation of platelet function with stimulation of relaxation of contractile cells and resulting vasodilatation [17, 19]. Therefore, inhibition of cGMP degradation by PDE5 inhibitors might preserve the cGMP pool, thus promoting the favorable action of NO and eventually attenuating the manifestations of I/R injury.

Sildenafil is a potent selective inhibitor of PDE5 and is widely being used for the treatment of erectile dysfunction in men. It has also been investigated in the context of persistent pulmonary hypertension with satisfactory results [20]. There is evidence that sildenafil is also capable of inducing a preconditioning-like effect in I/R injury of various tissues such as the heart, lung, kidney, and brain [21–24]. In spite of a notable number of studies on pharmacological strategies aiming at attenuating the manifestations of liver I/R injury, literature is scarce regarding the use of sildenafil in this context. Therefore, we designed this experimental study in order to evaluate the effect of sildenafil in a liver I/R rat model by using histopathological and biochemical parameters. In specific, we tested the hypothesis that sildenafil exerts a protective effect on the liver, as this has been evidenced for other tissues subjected to I/R insults.

2. Methods

2.1. Animals and Experimental Design. This protocol was approved by the Animal Research Committee of the University of Athens and the Committee of Bioethics of Aretaieion Hospital. Handling and care of the animals was in accordance with European guidelines for ethical animal research.

Forty male Wistar Rats weighing 300–350 g were used. The animals were housed in individual cages, at constant temperature conditions (21°C), with alternating 12-hour light/dark cycles. They were also maintained on a standard diet and water *ad libitum*.

The animals were randomly allocated into four groups: a group which received no treatment and underwent midline laparotomy only (sham group, $n = 10$), a group that underwent midline laparotomy only as the sham group after the administration of sildenafil 0.3 mg/kg 60 minutes before the operation (sild+sham group, $n = 10$), a group that was subjected to partial liver ischemia and reperfusion (I/R group, $n = 10$), and a group that underwent liver ischemia and reperfusion after the administration of sildenafil 0.3 mg/kg 60 minutes before the induction of hepatic ischemia (sild+I/R group, $n = 10$).

Sildenafil was administered at a dose of 0.3 mg/kg with an intraperitoneal injection. This dose is the equivalent of the dose used for the management of pulmonary hypertension [25–27].

2.2. Surgical Procedure. All animals were anesthetized with intraperitoneal administration of ketamine 100 mg/kg and xylazine 10 mg/kg. The rats had their abdomen clipped off hair and prepared with povidone-iodine solution. All subsequent procedures were performed using aseptic technique with sterile equipment and prostheses. The rats underwent a 4 cm midline abdominal incision through the musculature and peritoneum. Twenty IU/kg of heparin was administered intraperitoneally. After identification of all liver lobes, the portal vein was identified. In the I/R and the sild+I/R groups, the portal vein and hepatic artery were occluded with the use of atraumatic vascular clips, immediately after the bifurcation of the right lateral branch. The clamp was partial, aiming at blocking the portal venous and hepatic arterial blood supply to the median and left lateral lobes of the liver. This yielded approximately 70% of hepatic ischemia, which was maintained for a 45-minute period. We aimed at partial ischemia in order to avoid prolonged blood pooling in the splanchnic bed, which could result in splanchnic congestion and intestinal injury. For this reason, the gut was monitored macroscopically throughout the ischemic period for signs of portal hypertension. At the end of this period, the clamp was removed and portal and arterial blood flow were restored. Reperfusion was confirmed macroscopically by change of the color of the liver. In the sham and the sild+sham groups, only the hepatic pedicle was identified and hepatic vessel clips were not applied.

The ischemic period was followed by two hours of reperfusion. During this period the abdomen was closed in the midline. At the end of the reperfusion period the abdomen was reopened and blood samples were collected from the abdominal aorta. Biopsies were taken from the liver lobes that were subjected to ischemia.

Anesthesia was maintained throughout the experimental period with intraperitoneal administration of ketamine and

Table 1: Liver pathology scoring.

Hepatocellular necrosis	
<2 foci in every hepatic lobule	1
3–5 foci in every hepatic lobule	2
<5 foci in every hepatic lobule	3
Diffuse necrosis in zone 3	4
Presence of bridging necrosis	5
Extensive necrosis	6
Inflammatory infiltration of portal spaces	
Mild inflammation	1
Moderate inflammation	2
Severe inflammation	3

Table 2: Quantification of myeloperoxidase staining.

Positive cells for myeloperoxidase staining per three optic fields (40x magnification)	Score
0–20 cells	0
20–40 cells	1
40–60 cells	2
>60 cells	3

xylazine, while at the end of the experiment, the animals were sacrificed by exsanguination while on anesthesia.

2.3. Liver Function Tests. After collection, blood samples were centrifuged at 4000 rpm for 20 minutes and serum was stored at −70°C until analysis. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined in systemic circulation using the Dimension RXL analyzer (Dade Behring, Dupond, Delaware).

2.4. Histopathological Evaluation. The tissue samples from the ischemic liver lobes were fixed in 10% buffered formaldehyde solution, embedded in paraffin, and then cut in 3–5 μm sections. Sections were stained with hematoxylin-eosin under standard histological methods and were evaluated by light microscopy. A pathology scoring system which assessed hepatocellular liver necrosis with a five-point grading scale and inflammatory infiltration with a three-point grading scale was used, as shown in Table 1.

2.5. Myeloperoxidase Assay. Tissue samples from the ischemic liver lobes were stained immunohistochemically for myeloperoxidase (MPO). Briefly, formaldehyde-fixed, paraffin-embedded sections were incubated for 30 minutes at room temperature with 1% polyclonal rabbit antihuman myeloperoxidase antibodies, according to the manufacturer's instructions (Dako Pathology Products, Hamburg, Germany). MPO staining was quantified according to the expression of MPO in three optic fields (40x magnification) as shown in Table 2. All microscopic examinations were performed by an expert pathologist, who was unaware of the treatment group.

2.6. Measurement of Intercellular Adhesion Molecule-1 (ICAM-1) mRNA Levels. The expression of ICAM-1 was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). and afterwards, 5 μg RNA was used for complementary DNA synthesis. ICAM-1 gene expression was normalized with β-actin gene expression. RT-PCR was performed at the following temperatures: for ICAM-1, denaturation at 94°C for 45 sec, primary annealing at 55°C for 30 sec, and primer extension at 72°C for 90 sec and for β-actin, 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, respectively. The sequences of the primer sets were GAT GCT GAC CCT GGA GAG CA and CAG GGA CTT CCC ATC CAC CT for ICAM-1. Those for β-actin were TAT GGA ATC CTG TGG CAT CC and ACA GAA GCA ATG CTG TCA CC. Semiquantification of gene expression was performed and mRNA expression of ICAM-1 was presented as a percentage of β-actin.

2.7. Apoptosis Assay. Specimens 4 μ thick were also cut and stained with the TUNEL assay. This assay is performed by labelling the free 3′-OH ends of DNA strand breaks that are produced after DNA fragmentation during apoptosis, with fluorescent nucleotides in an enzymatic labelling method with terminal deoxynucleotidyl transferase. In our study a commercial kit Apoptosis-1,5 (YLEM, Rome, Italy) was used for the TUNEL assay, according to the manufacturer's instructions. Ten random fields were analyzed for each TUNEL-stained tissue sample. All slides were examined by the same pathologist, who was unaware of the treatment group. Data regarding TUNEL staining were expressed as mean ± SD percentage of nuclei containing apoptotic bodies per high-power field.

2.8. Statistical Analysis. Variables were tested for normality of distributions with the Kolmogorov-Smirnov test. AST and ALT values and ICAM-1 mRNA levels as a percentage of β-actin and percentage of apoptotic bodies were normally distributed and differences between experimental groups were analyzed with one way analysis of variance (ANOVA), followed by the Holm-Sidak test for *post hoc* comparisons between individual groups. Histology and MPO staining scores did not follow normal distribution and differences between experimental groups were analyzed with one Kruskal-Wallis ANOVA on ranks, followed by the Mann-Whitney U test for *post hoc* comparisons between individual groups. All calculations were carried out using SPSS 17.0 for Windows. The level of statistical significance was set to $P < 0.05$. Results are expressed as mean ± SD or as median (25th–75th percentiles) depending on normality of distributions.

3. Results

3.1. Serum AST and ALT Levels. I/R of the liver substantially increased serologic markers of hepatocyte injury. Specifically, following two hours of reperfusion, AST and ALT levels in the I/R alone and the sild+I/R groups were significantly

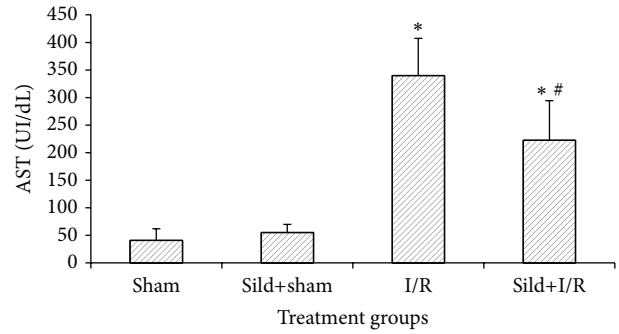


Figure 1: AST serum levels two hours after reperfusion; $^{*}P < 0.05$ in comparison to the sham group; $^{\#}P < 0.05$ in comparison to the I/R group.

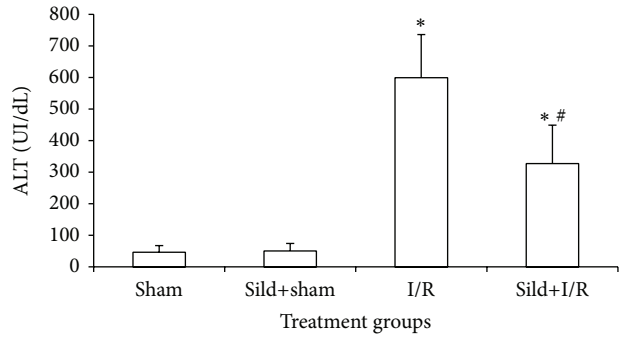


Figure 2: ALT serum levels two hours after reperfusion; $^{*}P < 0.05$ in comparison to the sham group; $^{\#}P < 0.05$ in comparison to the I/R group.

higher than in the sham and the sild+sham groups. However, sildenafil pretreatment attenuated hepatocellular injury, as the values of AST and ALT were significantly lower in the sild+I/R group, as compared to the I/R group (AST 222.7 ± 71.5 UI/dL versus 340.0±67.2 UI/dL, $P < 0.05$, Figure 1) (ALT 326.9 ± 122.3 UI/dL versus 599.1 ± 137.2 UI/dL, Figure 2).

3.2. Histopathological Analysis. Neither the sham group nor the sild+sham group showed any significant histological findings. Liver samples from the I/R group showed extensive areas of zone 3 necrosis. In addition, sinusoidal congestion, microthrombosis, eosinophilic degeneration with hepatocyte vacuolization, and neutrophil infiltration were present. Tissue samples from the sild+I/R group exhibited lower severity of lesions since foci of hepatocyte necrosis and leukocyte infiltration were markedly suppressed in comparison to the I/R group (Figures 3(a) and 3(b)). Morphological findings were further confirmed by the semiquantitative assessment, where the sild+I/R group had significantly lower scores of hepatocellular necrosis and inflammatory infiltration, as shown in Table 3.

3.3. Myeloperoxidase Staining. Animals subjected to liver I/R had increased expression of MPO activity in liver tissue compared to animals of the sham and sild+sham group and significantly higher expression of MPO activity as compared to animals of the sild+I/R group ($P < 0.01$) (Table 3).

3.4. Measurement of ICAM-1 mRNA Levels. Through assessment of mRNA transcripts, liver I/R injury remarkably increased mRNA expression of ICAM-1, while sildenafil pretreatment in the sild+I/R group attenuated I/R-induced mRNA expression (Table 3, Figure 4).

3.5. Evaluation of Hepatic Apoptosis by TUNEL Staining. Specimens from the sild+I/R group showed significantly fewer cells stained positive by TUNEL versus the I/R group (32% ± 9% versus 58% ± 15% percentage of nuclei containing apoptotic bodies; $P < 0.05$) (Table 3, Figure 5).

4. Discussion

In the present study we used a partial liver ischemia-reperfusion model in rats in order to simulate various aspects of hepatic surgery under vascular occlusion, including surgery for liver tumors, liver transplantation, and hepatic trauma. Previous studies have shown that inflammation, apoptosis, and altered microcirculation are histological findings evident even in the early stage of hepatic ischemia-reperfusion injury.

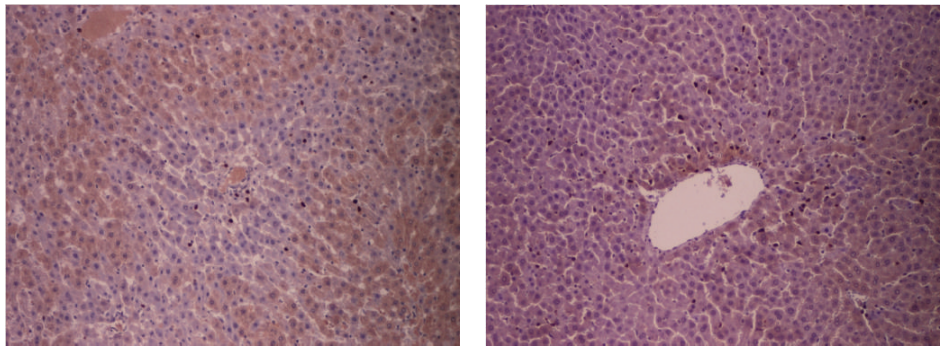
The results of our study suggest that sildenafil used as pretreatment has a hepatoprotective effect in a rat model of partial liver ischemia-reperfusion injury. This was evidenced by suppression of the increase in AST and ALT, decreased scores of necrosis, attenuation of morphological liver injury, and antiapoptotic activity. Decreased expression of ICAM-1 mRNA and reduction of leukocyte-endothelial interaction, as evidenced by attenuated MPO staining in the sildenafil-treated rats, were also observed.

In the past, several studies have investigated the role of NO in partial liver ischemia-reperfusion models with controversial results [14]. Whether NO has a protective or deleterious effect probably depends on the type of insult, the source and quantity of NO produced, and the cellular redox status of the liver [16, 28–31]. It is considered that endogenous (basal) NO, produced by an early and transient activation of eNOS, protects both hepatocytes and endothelial cells against reperfusion injury in the liver [32]. NO counteracts the vasoconstriction caused by endothelin-1, which is involved in microvascular dysfunction, particularly during the early stages of liver I/R [33]. For instance, it has been found that I/R injury is exacerbated in e-NOS deficient animal models [34, 35], whereas genetic overexpression of eNOS has been shown to attenuate hepatic I/R injury in a rat model [36]. Therefore, eNOS expression has a cytoprotective effect by maintaining basal levels of NO production and acts protectively against the early phase of I/R injury by preservation of the sinusoidal structure and maintenance of blood flow through the hepatic microcirculation, thus limiting the extent of I/R injury through a cGMP pathway [14, 16]. eNOS expression is downregulated during liver reperfusion as a result of inhibition of eNOS activity by oxidative stress and absence of flow within the sinusoids during ischemia [14].

Table 3: Effect of sildenafil pretreatment on histopathology scores, MPO staining, ICAM-1 mRNA levels (as a percentage of β-actin), and percentage of apoptotic bodies.

	Sham group ($n = 10$)	Sild+sham group ($n = 10$)	I/R group ($n = 10$)	Sild+I/R group ($n = 10$)
Histology				
Hepatocellular necrosis scoring	1 [1-1]	1 [1-1]	5 [5-6]*	2.5 [2-3]$^{*\#}$
Inflammatory infiltration scoring	1 [1-1]	1 [1-1]	3 [3-3]*	2 [1-2]$^{*\#}$
MPO staining scoring	0 [0-0]	0 [0-1]	3 [2-3]*	1 [1-1]$^{*\#}$
ICAM-1 mRNA levels (% of β-actin)	4.7 ± 1.1	5.2 ± 1.6	51.5 ± 23.3*	17.1 ± 5.6$^{*\#}$
Apoptotic bodies (%)	14 ± 5	15 ± 7	58 ± 15*	32 ± 9$^{*\#}$

Values are presented as mean ± SD for ICAM-1 mRNA levels and apoptotic bodies and as median [25th–75th percentiles] for histology and MPO staining scores.
$^{*}P < 0.05$ in comparison to the sham group; $^{\#}P < 0.05$ in comparison to the I/R group.



(a) (b)

Figure 3: (a) Hepatocellular necrosis in zone 3 of the hepatic lobule and increased inflammatory infiltration in the I/R group (hematoxylin-eosin ×100). (b) Few foci of hepatocellular necrosis and mild inflammatory infiltration in the sild+I/R group (hematoxylin-eosin ×100).

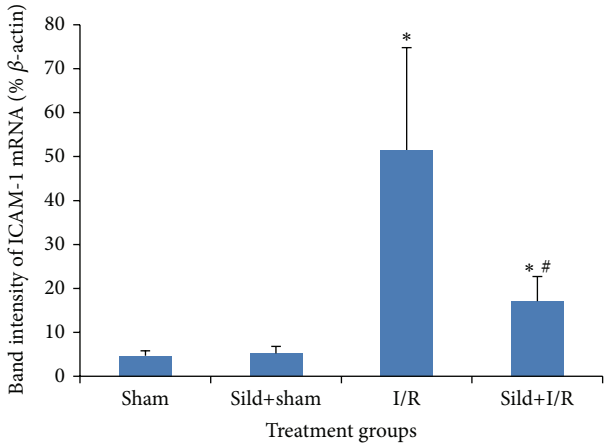
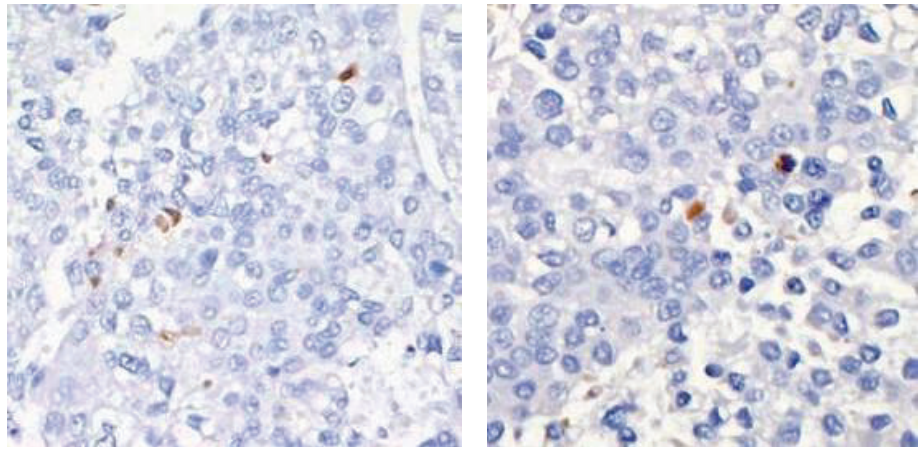


Figure 4: Band intensity of ICAM-1 mRNA as a percentage of β-actin; $^{*}P < 0.05$ in comparison to the sham group; $^{\#}P < 0.05$ in comparison to the I/R group.

The decreased production of NO from eNOS increases the vascular resistance of the intrahepatic circulation and contributes to the microcirculatory failure following reperfusion [8, 10]. Analogous findings in the microcirculation have also been shown in the context of liver cirrhosis. In particular, a decrease of NO-related relaxation response or a reduction in hepatic NO bioavailability mediated via a decrease in eNOS activity seems to be one of the mechanisms leading to the increased intrahepatic resistance in cirrhotic livers [37]. Similarly, increased expression of PDE-5, which may enhance the degradation of hepatic cGMP and be involved in the decreased vasodilator response to NO, has been observed in the intrahepatic vasculature of cirrhotic rat livers [38].

On the other hand, the induction of iNOS, stimulated by oxidative stress during reperfusion and expressed within 6 hours of reperfusion, seems to increase reperfusion-mediated liver injury [16, 17, 39]. Once i-NOS is induced, excessive amounts of NO are produced. In the presence of superoxide, NO can form peroxynitrite, a potent oxidant and protein nitrating agent and a substance extremely toxic to cells. Peroxynitrite subsequently can decompose to generate a strong oxidant with reactivity similar to hydroxyl radical [40]. Therefore, apart from favorable actions, NO, produced in excess, may also prove hazardous and have cytotoxic potential through its interaction with superoxide anion and contribute to hepatic injury accompanying the late phases or reperfusion. Under normal conditions, excessive NO production clearance is achieved through hemoglobin. However, postreperfusion microcirculation failure may lead to impaired clearance, further enhancing the deleterious effects of NO during reperfusion.

Sildenafil is a potent inhibitor of PDE5, which catalyses the breakdown of cGMP. Thus, the administration of sildenafil preserves NO-driven cGMP levels by reducing its



(a) (b)

Figure 5: Apoptosis recorded as the percentage of positively staining nuclei per high-power field was higher in the I/R group (a) as compared to the sild+I/R group (b). TUNEL staining ×200, apoptotic bodies appear brown in color.

degradation and enhancing its action as a potent vasodilator and an inhibitor of thrombocyte aggregation [17, 19]. cGMP activates protein kinase G, which in turn opens mitochondrial adenosine triphosphate potassium (mitoK$_{ATP}$) channels, conferring the protective effect against I/R injury [17]. Sildenafil has also been shown to upregulate eNOS expression, thus directly enhancing NO bioavailability [41, 42]. Moreover, there is substantial experimental evidence that activation of protein kinase C could be one of the intracellular signal transduction pathways controlling sildenafil-dependent cardiac protection in the rabbit heart [43]. A direct action of sildenafil on mitoK$_{ATP}$ channels has also been suggested to mediate the sildenafil-induced protection against ischemic injury [44]. Additionally, sildenafil has been shown to have a protective effect independent of the NO/cGMP pathway [45]. Finally, a direct neutralization of free radicals has been attributed to sildenafil [46].

Sildenafil has been used in the past in experimental models of ischemia-reperfusion of various organs such as heart, kidney, brain, and lung [21–24]. It has also been administered in pulmonary hypertension settings with favorable outcomes [20, 47]. Literature is very scarce, if any, regarding the use of sildenafil in the setting of liver I/R injury.

In the present study we chose to administer sildenafil at a dose of 0.3 mg/kg. This dose corresponds to the dose administered in studies of pulmonary hypertension and was chosen because it has been shown to be well tolerated with no significant hemodynamic instability [25–27]. Intraperitoneal administration was preferred over *per os* administration due to the characteristic intestinal and hepatic first pass effect, which decreases bioavailability to 14.6% when administered orally [48]. The rationale for using sildenafil to attenuate I/R injury, despite the known deleterious effects of iNOS induced NO production during reperfusion, is that a selective PDE5 inhibitor, given as a pretreatment prior to ischemia and reperfusion, could enhance the beneficial action of NO during the reperfusion-induced suppression of eNOS production. In addition, sildenafil metabolism and elimination would prevent any enhancement on the late stage of reperfusion when iNOS induction has been shown to be cytostatic and antiproliferative.

According to our results, in animals pretreated with sildenafil before the onset of hepatic ischemia, attenuation of hepatocellular necrosis and inflammatory infiltration was noted in contrast to animals of the I/R group which exhibited a greater degree of necrosis and inflammation under microscopic evaluation. These findings delineate the beneficial effects of sildenafil pretreatment at the cellular level. Similarly, markers of hepatocyte injury, that is, AST and ALT, were attenuated in animals pretreated with sildenafil. This suggests that sildenafil may attenuate oxidative aggression-induced tissue injury through hepatocyte and sinusoidal endothelial cell membrane stabilization and minimization of intracellular calcium overload or through improvement of sinusoidal blood flow. The beneficial action of sildenafil administration in the microvascular level and the sinusoids has also been demonstrated in cirrhosis. In an experimental study, acute incubation of sildenafil increased the vasodilator response to NO in cirrhotic rat livers [38]. In a human study, acute administration of sildenafil increased hepatic production of NO and cGMP and decreased the hepatic sinusoid resistance in cirrhotic patients [49]. Chronic sildenafil administration also seems to have beneficial effects. In particular, in an animal study, one week of sildenafil treatment enhanced NO bioavailability and contributed to the attenuation of intrahepatic resistance in cirrhotic rat livers [50]. In that study, it was shown that the administration of sildenafil upregulated the hepatic protein expression of eNOS and increased intrahepatic NO production. Additionally, PDE-5 levels of sildenafil-treated cirrhotic livers were significantly reduced. Finally, a significant increase in sinusoid area indicating increased vasorelaxation of sinusoids and enhanced volumetric flow was demonstrated by the use of fluorescent microscopy and microcirculatory analysis. It was thus concluded that the NO/cGMP pathway is augmented in the hepatic microcirculation, which elicits vasorelaxation and increases intrahepatic

blood flow [50]. Both the increased NO and inhibition of PDE-5 by sildenafil could contribute to the increase of hepatic cGMP levels. It has also been shown that high flow laminar shear stress increases production of NO though activation of eNOS [51, 52]. It can thus be postulated that the sildenafil-mediated increase of cGMP with the resulting increase in flow shear stress in the hepatic microcirculation contributes to the increased hepatic production of NO by sildenafil. The favorable effect of sildenafil in the microcirculation has also been demonstrated in the pulmonary and brain vasculature. Tantini et al. showed an antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells via inhibition of platelet derived growth factor-mediated activation of signal transduction pathways [53]. Additionally, Rosengarten et al. showed a beneficial effect of sildenafil on cerebral vascular reactivity [54]. Similar favorable vascular responses could underlie the improved histopathological and biochemical profile in animals pretreated with sildenafil before initiation of I/R in our experimental study. The hepatoprotection afforded by sildenafil could to a certain extent be also related to hepatoproliferative properties that have been attributed to sildenafil and its downstream signaling pathway. In particular, sildenafil promoted hepatocellular regeneration in a rat model of liver injury caused by chronic ethanol feeding, manifested by a greater mitotic index of liver cells in sildenafil-treated rats [55]. In addition, sildenafil administration in a rat model of partial hepatectomy accelerated regeneration rate of the remnant mass, as expressed by cell proliferation markers and mitotic counts [56]. The beneficial effects of sildenafil on hepatic regeneration at the cellular level have recently been demonstrated in a paracetamol-induced hepatotoxicity rat model [57].

Moreover, MPO staining in the sild-I/R group was significantly attenuated as compared to the nonsildenafil-treated animals. MPO is a neutrophil-specific enzyme, which is secreted during polymorphonuclear accumulation and is used as an index of hepatic leukocyte infiltration [58, 59]. As already mentioned, during ischemia, neutrophils accumulate in the endothelium and such accumulation may be markedly accelerated following reperfusion. Activated neutrophils release a variety of cytotoxic substances interacting with the endothelium and thereby causing tissue damage [60]. Moreover, aggregated neutrophils can physically obstruct capillary flow, causing further ischemia of the tissue and, lastly, they release large amounts of ROS, which contribute to the oxidative injury associated with hepatic I/R injury [61]. Therefore, as MPO activity is directly proportional to the neutrophil count, reduced MPO staining in the sildenafil-pretreated animals could be attributed to the attenuated neutrophil migration and activation during reperfusion.

Decreased expression of ICAM-1 mRNA with sildenafil administration was also observed. ICAM-1 is one of the adhesion molecules that are known to play an important role in the process of leukocyte-endothelial cell interaction. The adhesion of leukocytes to the microvascular endothelium is a manifestation of I/R injury and is mediated by a variety of cell-surface molecules like ICAM-1. This molecule is present at low levels on most endothelial cells and is upregulated in case of inflammation and I/R injury [10, 62]. In fact, blocking its activity with monoclonal antibodies has been found to protect against I/R injury [63, 64]. Moreover, NO donors have been found to attenuate leukocyte-endothelial cell reaction and to minimize the adhesive interactions between leukocytes and the endothelial cell surface, thus maintaining vascular patency [65–67]. Therefore, it is possible that sildenafil, through its NO downstream pathway, minimizes those adhesive interactions between leukocytes and the endothelial cell surface and, together with suppression of neutrophil tissue migration and infiltration, as shown with the attenuation of MPO activity, preserves vascular permeability and improves hepatic microcirculation.

Regarding apoptotic response, the sildenafil-pretreated group showed significantly fewer cells positive to TUNEL staining in comparison to the animals of the I/R only group. Although an excessive inflammatory response and necrosis are widely considered to be the major characteristic in the process of liver damage after I/R injury, there is also evidence that cell apoptosis could also be a primary mechanism of damage [11, 12, 68]. It could be possible that some of the protective effects elicited by sildenafil could be conferred through modulation of the apoptotic response. Sildenafil has been shown to directly protect adult cardiomyocytes against apoptosis following I/R injury in mice models [42, 69, 70]. It has also been shown that physiologically stimulated by NO soluble guanylate cyclase inhibits apoptosis [71]. Another study has suggested that eNOS-derived NO might assist in decreasing the percentage of apoptotic cells during hypoxia/reoxygenation in a lung model [72]. In addition, a report by Akao et al. has shown that openers of the mitoK_{ATP} channel were able to reduce apoptosis induced by oxidative stress in neonatal rat cardiomyocytes [73]. Therefore, we could hypothesize that a NO-cGMP signaling pathway could underlie the protective effect of sildenafil against hepatic apoptosis through its mitoK_{ATP} channel opening properties, as this has already been demonstrated in a rabbit heart model [44]. Our study is the first to demonstrate an antiapoptotic effect of sildenafil in experimental liver I/R injury through TUNEL staining. Further studies are required though to delineate the exact antiapoptotic pathways involved in sildenafil action.

Our study carries certain limitations, including the brief monitoring period as well as the fact that there was no hemodynamic monitoring of the animals during our experiment. Moreover, we studied a single dose of sildenafil, so we can only speculate about effect of different dosing regimens.

In conclusion, the results of this preliminary report suggest that sildenafil seems to attenuate hepatic ischemia-reperfusion injury when administered intraperitoneally prior to liver ischemia according to morphological and functional criteria, with preischemic administration mimicking the physiological phenomenon of preconditioning. This concept needs to be further investigated using different dosage schemes, administration times, and duration of treatment in order to extrapolate the results of this experimental study to realistic clinical environments and in order to be able to evaluate the sildenafil-mediated protection of hepatocytes during liver transplantation and in the setting of major liver

resections, where metabolic and energy support of a small liver remnant could be of great importance. In addition, sildenafil administration could also be investigated in a posttreatment fashion, since there may be clinical occurrences where the onset of reperfusion is more predictable and it may be more feasible to initiate administration at reperfusion rather than before the ischemic event. Finally, future studies should include hemodynamic monitoring of sildenafil administration in the setting of liver I/R injury and also evaluate the exact mechanism of sildenafil action on apoptosis pathways.

Conflict of Interests

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

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Review Article

Functional Roles of Protein Nitration in Acute and Chronic Liver Diseases

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Nitric oxide, when combined with superoxide, produces peroxynitrite, which is known to be an important mediator for a number of diseases including various liver diseases. Peroxynitrite can modify tyrosine residue(s) of many proteins resulting in protein nitration, which may alter structure and function of each target protein. Various proteomics and immunological methods including mass spectrometry combined with both high pressure liquid chromatography and 2D PAGE have been employed to identify and characterize nitrated proteins from pathological tissue samples to determine their roles. However, these methods contain a few technical problems such as low efficiencies with the detection of a limited number of nitrated proteins and labor intensiveness. Therefore, a systematic approach to efficiently identify nitrated proteins and characterize their functional roles is likely to shed new insights into understanding of the mechanisms of hepatic disease pathophysiology and subsequent development of new therapeutics. The aims of this review are to briefly describe the mechanisms of hepatic diseases. In addition, we specifically describe a systematic approach to efficiently identify nitrated proteins to study their causal roles or functional consequences in promoting acute and chronic liver diseases including alcoholic and nonalcoholic fatty liver diseases. We finally discuss translational research applications by analyzing nitrated proteins in evaluating the efficacies of potentially beneficial agents to prevent or treat various diseases in the liver and other tissues.

1. Introduction

Nitric oxide (NO) is a common free radical that is synthesized via enzymatic and nonenzymatic reactions in various cells and tissues. NO is also a very important intracellular signaling molecule in all vertebrates and even in plants. NO is enzymatically synthesized through three different isoforms of NOS (nitric oxide synthase), namely, NOS1, NOS2, and NOS3 [1]. Neuronal NOS (nNOS or NOS1) is expressed in the brain in large quantities [2, 3]. NOS2, known as inducible NOS (iNOS), is induced in various tissues in response to proinflammatory cytokines or oxidative stress under pathological conditions or following exposure to toxic agents [4]. NOS3, known as endothelial NO synthase (eNOS), is typically expressed in vascular endothelial cells and associated to plasma membrane [4]. Thus the three NOS isozymes were named either after their constitutive expression in certain tissues (nNOS and eNOS) or after their expression mechanism (iNOS). While both NOS1 and NOS3 can be activated by intercellular calcium and calmodulin, NOS2 can be induced completely with normal levels of calcium [4]. NO is produced by a reaction that is composed of two sequential steps: (1) the hydroxylation of guanidino nitrogen of L-arginine, leading to the generation of the intermediate N^{ω}-hydroxy-L-arginine (NOHA), and (2) NOHA is then oxidized to NO and L-citrulline [5]. NO by itself is not highly reactive since NO is the intermediate molecule between molecular oxygen (O_2) and nitrogen (N_2) [4]. NO has a very short half-life; however, it can diffuse freely across cell membranes [6]. The classical NO signaling is related to its activation of soluble guanylate cyclase (sGC) and subsequently cyclic guanosine monophosphate- (cGMP-) dependent protein kinases [7].

The less- and nonclassical NO signaling pathways are related to NO binding to cytochrome c oxidase in the mitochondria and the cGMP-independent NO-related posttranslational modifications (PTMs), respectively [7].

There are many forms of NO which are interchangeable and leading to the production of reactive nitrogen species (RNS) such as nitrosonium cation (NO^+), nitroxyl radical ($NO^·$), nitroxyl anion (NO^-), nitrite (NO_2^-), nitrate (NO_3^-), and nitrous oxide (N_2O). These intermediates provide the NO molecule as a unique signaling molecule that can act within the cell or interact with adjacent cells without the need of a receptor [6]. The generation of secondary oxides of nitrogen such as peroxynitrite ($ONOO^-$) and nitrosothiols (RSNO) can also produce diverse biological effects through interactions with cellular macromolecules (e.g., DNA, lipids, and proteins), as illustrated in Figure 1. Peroxynitrite can stimulate nitration of tyrosine (Tyr) residue(s) and *S*-nitrosylation of Cys residues, both of which can lead to alterations of protein structure and function [8, and references therein]. Nitrogen dioxide (NO_2) can also interact with other oxidants such as superoxide radicals ($O_2^{·-}$), H_2O_2, and transition metal centers in various heme-containing proteins, leading to production of peroxynitrite, which nitrates Tyr residues of various proteins generating 3-nitroTyr (3-NT), which is widely accepted as a foot print of peroxynitrite formation [9–11]. Reactive oxygen species (ROS) including superoxide radicals can be produced from various sources including mitochondrial electron transport chain (ETC) and other cellular enzymes such as NADPH oxidase and myeloperoxidase or eosinophil oxidase in immune cells (i.e., macrophage cells and neutrophils), ethanol-inducible cytochrome P450 2E1 (CYP2E1) and CYP4A isozymes in endoplasmic reticulum (ER), cytosolic xanthine oxidase, and so forth [12–19]. RNS not only interact with Tyr, but also with tryptophan (Trp) [20], lipids [21], and vitamins [22, 23]. Thus, the nitrative modifications of target proteins, DNA, lipids, and vitamins, usually contributing to alterations of their normal functions and the development or progression of tissue injury [4, 24, 25]. Tyr is a common amino acid found in most proteins [26] and is readily an accessible target for protein nitration since it is often exposed on the surface of the protein due to its mild hydrophilic characteristic [27, 28]. Thus, protein nitration data in literature is largely associated with Tyr nitration and functional consequences. The modification of Trp residues in proteins may occur in a more limited number of sites than that of Tyr residues since Trp is less abundant than Tyr in the protein and is usually buried inside with an exception for a few surface exposed Trp residues. Therefore the modification of those Trp residues may alter specific interaction of proteins and/or enzymes with other molecules, which may cause functional dysregulations [20]. However, in this review, we do not discuss much about Trp nitration since its formation, identification, and significance were extensively reviewed elsewhere [20, 29, 30]. It is likely that modification of each amino acid (e.g., nitration of Tyr or Trp or *S*-nitrosylation of Cys) seems to depend on the local microenvironment such as pH, solvent exposure or accessibility, peptide loop structure, and the presence of other competing amino acids (e.g., Cys near the potentially nitrated Trp or Tyr) [20] or a denitrase present in several tissues [31].

Protein Tyr nitration has been reported in correlation with many pathological conditions such as cardiovascular disorder, diabetes, hepatic disease, ischemia-reperfusion (I-R) injury, neurodegenerative diseases, stroke, inflammatory diseases, and cancer, as reported in many human diseases and experimental models including cell culture systems [4, 32]. Despite the numerous reports about the occurrence and consequence of Tyr nitration in various human diseases and in experimental animal models [4, and references therein], there have been a relatively small number of reports that systematically dealt with identification and functional characterization of nitrated proteins in various subcellular organelles, including mitochondria, especially under conditions with increased nitroxidative stress. Fewer reports on protein oxidation, nitrosation, and nitration might be due to the requirement for specific reagents, the lack of suitable methods to systematically identify and purify nitrated proteins, and the relatively late development and advancement of highly sensitive mass spectrometry (MS) instruments. There are many questions that need to be addressed when working with nitrated proteins as follows: (1) what are the sources of ROS/RNS in enhancing protein nitration? (2) which proteins are nitratively modified? (3) are their activities/functions altered following nitrative modifications? (4) what are the overall implications of nitrated proteins in mitochondrial dysfunction or other organelles in various disease states including hepatic liver disease/injury? (5) can the nitrative protein modifications and subsequent dysfunction be prevented with a potential therapeutic agent? and (6) most importantly, do we have a suitable method to efficiently identify the nitrated proteins to study the causal relationship between protein nitration and mitochondrial dysfunction and tissue injury? In this review, we discuss the roles of nitrative stress and protein Tyr nitration in the development and/or progression of various stages of liver disease. We specifically focus on nitration of mitochondrial proteins and their functional implications. We also briefly discuss various methods for identifying nitrated proteins in experimental models with emphasis on the immunoaffinity purification of nitrated proteins followed by their identification by MS analysis. Finally we briefly discuss the utility of studying nitrated proteins in different subcellular fractions in various tissues as well as future translational research opportunities.

2. Functional Consequences of Protein Nitration in Acute and Chronic Liver Diseases

Many pathological conditions usually result from changes in genetic or environmental factors or a synergism between both factors. Increased nitroxidative stress seems to play a critical role in mediating the pathological effects of this synergism or even each factor alone (Figure 1). Under normal physiological conditions, protein nitration and function alterations could be properly managed by the antioxidant host defense system [50, 51] increased nitroxidative stress, four possible outcomes

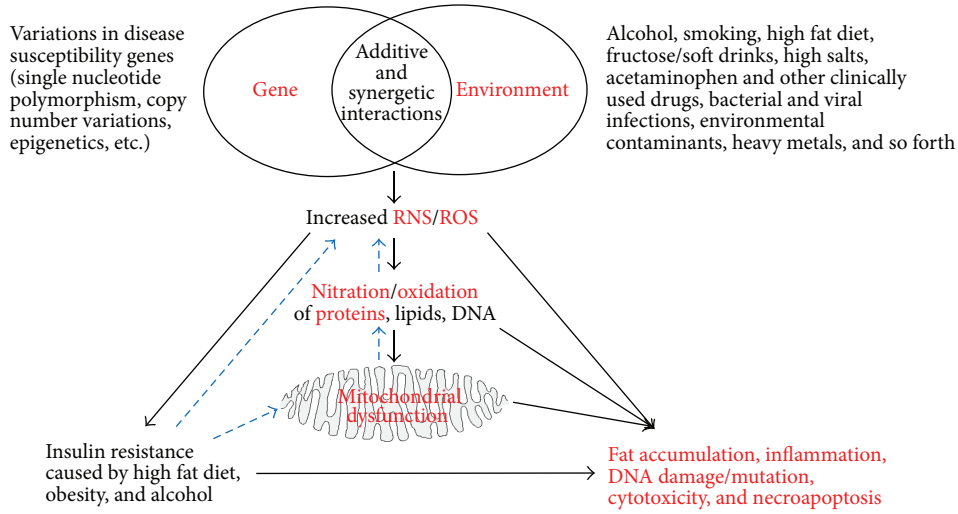

Figure 1: Synergistic interaction between genetic and environmental factors in promoting acute and chronic liver diseases. Additive or synergistic interactions between genetic and environmental factors such as alcohol, smoking, fat diet, and other potentially toxic substances result in increased production of RNS/ROS, which can modify cellular DNA, lipids, and proteins, promoting mitochondria dysfunction and interrupting many important signaling pathways. Continued presence of increased nitroxidative stress (through a vicious cycle shown in blue dotted arrows) contributes to acute and chronic liver diseases including alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD).

may arise from protein nitration: (A) a decrease or loss, (B) an increase, (C) immunomodulation and immunogenicity, or (D) little or no effect on the biological functions of the modified target proteins. One of the challenges that arise when determining the functional implications of protein nitration is that nitration is not unique to Tyr residues since Trp can be also nitrated. In addition, various amino acids such as Trp, cysteine (Cys), histidine (His), proline (Pro), lysine (Lys), and methionine (Met) can be also oxidized or *S*-nitrosylated [20, 50]. Another challenge could be to identify and characterize nitrated proteins that are expressed in very low quantities such as various transcription factors and signaling proteins, contributing to tissue injury.

2.1. Inhibition of Nitrated Proteins and Functional Consequences. The decreased function by protein nitration may result from the inhibition or loss of catalytic activity and/or decreased protein levels since protein nitration likely changes the protein secondary structure, hindering the access of the substrate to the activity site [4]. Alternatively, nitrated and/or oxidatively modified proteins that are improperly folded or damaged are known to be removed through ubiquitin-dependent proteolytic degradation mechanism [52, 53] while trypsin, serine proteases, or calpain is not involved in this process [53]. In addition, nitration and/or oxidation may lead to protein aggregation and the consequent loss of its activation and/or functions reported with α-synuclein, a major constituent of the typical protein aggregates observed in several neurodegenerative diseases that are collectively referred to as synucleinopathies [54, 55]. The removal of nitrated proteins can serve as a defense mechanism against nitroxidative stress-related harmful consequences but also plays an essential role in many vital processes including cell division, apoptosis, cell differentiation, DNA repair, membrane transport, oncogenesis, and signal transduction [56, 57]. At the same time, degradation with decreased levels of essential proteins for cell maintenance and survival can also be detrimental to the cells, especially when the degradation rates of proteins essential for energy production, antioxidant or anti-inflammatory defense, urea metabolism, and so forth, exceed those of their regeneration or other compensatory mechanism(s) due to persistence of toxic effects.

By using biotin-*N*-maleimide (biotin-NM) as a sensitive biotin-switch probe to proficiently identify oxidized proteins, we previously showed that many mitochondrial proteins including the enzymes involved in fat oxidation and energy supply could be oxidatively modified under increased nitroxidative stress and thus inactivated, leading to increased fat accumulation and ATP depletion in the experimental models of alcoholic fatty liver disease (AFLD) and acute I-R liver injury (Figure 1). We expect that protein nitration would also produce similar or more damaging effects in combination with other PTMs. We briefly describe the inhibition of catalytic activities and/or degradations of essential proteins following nitration and their functional implications in three major liver disease/injury models, namely, (1) AFLD, (2) nonalcoholic fatty liver disease (NAFLD), and (3) drug/xenobiotic-induced acute liver injury.

2.1.1. Alcoholic Fatty Liver Disease. The AFLD ranges from simple steatosis with microvesicular fat accumulation to more severe forms including steatohepatitis, fibrosis, cirrhosis, and finally hepatocellular carcinoma following chronic heavy alcohol ingestion [16, 58–62]. Obesity, hyperlipidemia, inflammation, nitroxidative stress, insulin resistance, and so forth are risk factors for AFLD ([63, 64] and references

therein). Several mouse models have been used to evaluate the effect of protein nitration on nitroxidative stress-mediated in AFLD. For instance, the role of protein nitration has been studied in mouse strains with ablated genes that are involved in either decreasing or increasing the levels of superoxide and NO. The mouse strains include *Cyp2e1*(−/−), SOD1(−/−), *SOD2*(−/−), *iNOS*(−/−), and TNF-α receptor (*TNFR*) (−/−), as described below.

Peroxynitrite and protein nitration were suggested to be main causes of acute and chronic alcoholic fatty liver injury models. Expression of iNOS was increased following the exposure to ethanol [15, 16, 37, 65–67]. Administration of the Lieber-DeCarli ethanol liquid diet has significantly increased the levels of steatosis, apoptosis, necrosis, and inflammation in wild-type (WT) mice compared to the corresponding *iNOS*(−/−) mice. The severity of liver injury was proportional to the levels of hepatic nitration and inhibition of mitochondrial function in alcohol-exposed WT mice, while *iNOS*(−/−) mice with markedly decreased levels of nitrated proteins were resistant to AFLD [15, 16] and that protein nitration was shown to inhibit complex I (NADH ubiquinone oxidoreductase) and complex V (ATP synthase) activities in acute and chronic alcohol-exposure models [36, 37, 68]. Thus protein nitration seems to play an important role in promoting the ethanol-mediated mitochondrial dysfunction and liver injury since these proteins are essential for normal mitochondrial function while protein nitration might lead to irreversible modification of the respiratory chain proteins, contributing to increasing mitochondrial sensitivity to NO and ultimately AFLD [69]. We and other laboratories clearly showed that nitration of ATP synthase led to significant inhibition of its activity in ethanol fed rats and mice [37, 70]. The suppression of ATP production would certainly increase mitochondrial sensitivity to other oxidative insults and necrotic injury. In an acute binge ethanol model, iNOS expression, serum nitrite/nitrates, increased protein nitration of mitochondrial complex V (ATP synthase), decreased activities of both complex I and V, and mitochondrial DNA depletion were observed in WT mice but not in *SOD2*(−/−) mice [71]. The authors suggested that these damaging effects were probably due to protein nitration since administration of iNOS inhibitors and peroxynitrite scavengers like uric acid ameliorated the ethanol-induced nitration, inhibition of activity, and mitochondrial depletion of ATP synthase. In addition, mice lacking SOD2, which would scavenge superoxide and thus block the peroxynitrite formation, exhibited prolonged mitochondrial DNA depletion while mice overexpressing SOD2 showed opposite outcomes [36]. Similar to the protective role of mitochondrial SOD2, cytosolic SOD1 also exhibits protective roles against ethanol-mediated hepatic damage. It has also been shown that, in mice deficient in SOD1, the levels of protective hepatic ATP content and SOD2 expression were decreased while oxidative damage and nitro-Tyr formation were elevated in response to ethanol feeding, leading to greater hepatic injury in the *SOD1*(−/−) mice [61]. This data suggests that mitochondrial dysfunction in the liver following ethanol exposure might originate from the compromise in the cytosolic antioxidant defense mechanism. In alcohol consumption studies, it was suggested that ethanol increased the sensitivity of mouse and rat hepatocytes to the combined effects of ethanol and NO [36, 71–73]. This combined effect of ethanol and NO increases the susceptibility to hypoxia and depression of mitochondria bioenergetics reserve energy state of the liver, leading to energy depletion and ultimately the development of alcohol-mediated hepatic injury [72, 73]. While NO donors inhibited mitochondria respiration and increased mitochondrial dysfunction in ethanol-fed rats more than the controls [73], *iNOS*(−/−) exhibited less severe liver injury with decreased levels of hypoxia-inducible factor 1-α in the perivenular region of the liver lobule than in the corresponding WT [16, 72]. Taken together, hepatic mitochondria from ethanol-fed mice or rats are more sensitive to NO and RNS while iNOS plays an essential role in determining the response to hypoxic stress in vivo. Since ethanol hepatotoxicity was also significantly prevented through a mechanism that involves a decreased inflammatory response, tumor necrosis factor-α (TNF-α) formation, and fatty liver [15], it was not surprising that *TNFR*(−/−) mice exhibited significantly less severe ethanol-mediated hepatotoxicity accompanied with markedly lower levels of protein Tyr nitration than those of their WT counterparts.

In addition, the elevated nitroxidative stress and subsequent hepatotoxicity in acute and chronic alcohol exposure models are correlated with the increased hepatic amounts and catalytic activities of CYP2E1, the most relevant cytochrome P450 for the development and progression of AFLD [13, 74–80]. For instance, in the chronic alcohol-fed mouse models, the levels of protein nitration were highest in *Cyp2e1* knock-in mice, followed by WT mice and lastly in *Cyp2e1*(−/−) mice, which exhibited almost basal levels of nitrated proteins [81]. The levels of protein nitration correlated well with the increased levels of hepatic transaminases, steatosis, and necrosis [81]. Since CYP2E1 is highly expressed in the endoplasmic reticulum (ER) and mitochondria [82–85], where iNOS is also present, it is conceivable to predict that protein nitration and oxidation can take place in both organelles and their participation in the development of AFLD either through increased ER stress and/or mitochondrial dysfunction, both of which are known as causative factors of AFLD.

Formation of peroxynitrite and protein nitration might not be the only causes for ethanol-induced hepatic toxicity. For instance, induction of nitrative stress along other forms of oxidation in intestinal epithelium may also serve as a cause for ethanol-induced gut leakiness and endotoxemia, leading to inflammatory liver injury. The intestinal epithelium, under normal conditions, serves as a highly selective barrier so that potentially toxic substances or products (e.g., lipopolysaccharide (LPS)) of the gut bacteria into the circulation [86–88]. Disruption of the intestinal barrier integrity (i.e., leaky gut) may lead to the penetration of luminal bacterial products such as endotoxin into the mucosa, then into the systemic circulation, and initiate local inflammatory processes in the intestine, blood vessels, and even in the liver [88]. Numerous studies in the literature showed that ethanol can compromise the integrity of intestinal epithelium, leading to increased gut leakiness in rodents [89–95] and human alcoholics [90, 96]. Ethanol-induced production of NO via induction of iNOS

Table 1: List of confirmed nitrated hepatic proteins and functional consequences.

Nitrated proteins	Activity	Hepatic indication (observed or expected)	References
Carbamoyl phosphate synthase-1 (CPS-1)	Decrease	Hyperammonemia, hepatic encephalopathy	[33]
Glutamine synthetase (GS)	Decrease	Hyperammonemia, hepatic encephalopathy related to sepsis	[34]
3-Ketoacyl-CoA thiolase (Thiolase)	Decrease	Decreased β-oxidation of fatty acids with increased hepatic steatosis	[35]
Aldehyde dehydrogenase 2 (ALDH2)	Decrease	Accumulation of acetaldehyde and lipid peroxides with increased aldehyde-related liver toxicity	[35]
Complex I (NADH ubiquinone oxidoreductase)	Decrease	ROS leakage, contributing to decreased energy production and increased apoptosis or necrosis	[36]
Complex V (ATP synthase)	Decrease	Decreased energy production with increased sensitivity toward necrotic liver injury	[11, 35–38]
Cytochrome p450 2E1, B6 (CYP2E1, CYP2B6)	Decrease	Drug metabolism: ROS production and ethanol- and drug-induced liver toxicity	[39]
Cytosolic Cu/Zn-SOD (SOD1)	Decrease	Decreased antioxidant defense with increased drug- or toxin-mediated hepatic damage	[35, 40]
Mitochondrial Mn-SOD (SOD2)	Decrease	Same as above	[35, 41]
Glutathione peroxidase (GPX)	Decrease	Same as above	[35]
Glutathione reductase (GR)	Decrease	Increased oxidative stress with elevated levels of oxidized glutathione	[42, 43]
AKT, IRβ, IRS-1, IRS-2	Decrease	Decrease insulin signaling with increased hepatic insulin resistance and fatty liver	[44]
CD95	Decrease	Increased hepatic anti-inflammatory defense	[45]
List of nitrated mitochondrial and cytosolic proteins	Not confirmed*	Not confirmed but likely contributing to mitochondrial dysfunction, ER stress, and liver damage	[35]
List of nitrated mitochondrial proteins	Not confirmed	Not confirmed but likely contributing to mitochondrial dysfunction and liver damage	[46]
List of nitrated proteins in different compartments	Not confirmed	Not confirmed but likely contributing to mitochondrial dysfunction, ER stress, and liver damage	[47]
Glutathione-S-transferase (GST)	Increase	Increased hepatic antioxidant defense	[41]
Heat shock protein 90 (Hsp90)**	Increase	Conversion to a toxic protein, contributing to increased liver toxicity	[48]
Protein phosphatase type 2A (PP2A)**	Increase	Increased microvascular endothelial permeability	[49]

*With the exception of the five proteins characterized in detail, as in the reference [35].
**Not confirmed in liver, but expected to occur.

and subsequent formation of peroxynitrite in intestinal Caco-2 cells lead to barrier dysfunction probably due to oxidation and nitration of cytoskeletal proteins and/or tight junction proteins [88, 90]. This data was also validated in vivo studies in a mouse model fed ethanol for 10 weeks with iNOS inhibitors. Coadministration with iNOS inhibitors attenuated ethanol-mediated NO overproduction, oxidative tissue damage, leaky gut, endotoxemia, and liver injury including steatosis [97].

CYP2E1 protein expression was also increased in Caco-2 intestinal epithelial cells and in both acute and chronic ethanol-exposed mouse models [78, 98]. In addition to increased nitroxidative stress and protein nitration, expression of redox-sensitive circadian clock proteins CLOCK and PER2 was significantly elevated in Caco-2 cells and in mice exposed to ethanol, leading to increasing intestinal hyperpermeability [98]. The usage of a CYP2E1 siRNA in Caco-2 cells or antioxidant *N*-acetyl cysteine (NAC) in mice exposed to ethanol ameliorated nitroxidative stress including iNOS expression and consequently inhibited the expression of CLOCK and PER2 as well as gut permeability. Further, recent studies from our laboratory showed that iNOS and protein nitration were also increased in intestinal epithelial cells in binge ethanol-exposed WT but not in the corresponding *Cyp2e1*(−/−) mice, leading to increased endotoxemia and subsequently inflammatory liver damage and apoptosis in ethanol-exposed WT mice [78]. We then showed that inhibition of iNOS induction and protein nitration of intestinal epithelial cells in mice pretreated with a CYP2E1 inhibitor or an antioxidant NAC ameliorated all the damaging effects in intestinal epithelial cells accompanied by decreased serum endotoxin levels and oxidative hepatic injury including steatosis and apoptosis in the ethanol-exposed WT mice. In contrast, these ethanol-mediated events were markedly attenuated in alcohol-exposed *Cyp2e1*(−/−). These results support at least a partial role of intestinal protein nitration in mediating the alcohol-induced gut leakiness and subsequent hepatic injury. Protein nitration in intestine cells is also likely to affect the cytoskeleton protein architecture [90] and/or intestinal tight gap-junction proteins, leading

to alteration of barrier function with increased permeability [78, 97]. Collectively, all these studies suggest that elevated iNOS and CYP2E1 play an important role, at least partially, in producing peroxynitrite and protein nitration in gut leakiness and AFLD, while normal levels of SOD1 and SOD2 seem protective against the development of AFLD since ethanol-mediated hepatic toxicity was worsened upon the inhibition or deletion of these SOD proteins. In addition, the development of peroxynitrite and subsequent protein nitration of essential mitochondrial proteins and other vital proteins in other subcellular organelles (e.g., cytosol) would be expected to play a causal or contributing role in mediating ethanol-mediated hepatotoxicities (Table 1). This data also suggests that in liver exposed to toxic substances/xenobiotics that increase ROS/RNS would sensitize the liver for even stronger and faster development of ethanol-mediated damaging effects, as evidenced with concurrent exposure to LPS, acetaminophen (APAP, a major ingredient of Tylenol), nicotine (a major component of smoking), 3,4-methylendioxymethamphetamine (MDMA) ([38, 99] and references within), high fat diet (HFD), and so forth. However, other forms of PTM including lipid peroxidation, *S*-nitrosylation including glutathionylation, glycosylation, and glycation including advanced glycation end-products (AGE), might also be involved in AFLD [8, 100] (Figure 1).

2.1.2. Nonalcoholic Fatty Liver Disease. NAFLD, usually caused by chronic ingestion of nonalcoholic substances such as HFD, cholesterol-containing fast food western diet, fructose, and choline-deficient diet, is one of the most common chronic liver diseases in the USA and developed countries [101, 102]. NAFLD often associated with overeating and obesity starts as simple steatosis and can progress to inflammation (nonalcoholic steatohepatitis, NASH), fibrosis, cirrhosis, and even cancer, as similar to the progression of AFLD [13, 103]. Many risk factors are known to promote the progression of NAFLD to NASH, fibrosis, and hepatocarcinoma. The risk factors include increased nitroxidative stress, mitochondria dysfunction, insulin resistance, disturbance of fat homeostasis, gut leakage, cytokine, and immune dysregulation [103, 104]. There are numerous reports in the literature showing that increased levels of iNOS, CYP2E1, and protein nitration play an important role, at least partially, in the development and/or progression of NAFLD, as shown in various experimental models and people with NASH ([74, 105–115], and references therein). In a NASH model fed a HFD for 20 weeks, the overexpression of tissue inhibitor of metalloproteinase (TIMP3) in macrophages ameliorated HFD-induced insulin resistance, adipose inflammation, and NAFLD probably through the inhibition of various parameters of nitroxidative stress including hepatic protein nitration [116]. The role of leptin in mediating the progression of NAFLD to NASH was evaluated in WT and leptin-deficient *ob/ob* mice fed a HFD for 16 weeks followed by treatment with carbon tetrachloride (CCl4) [117]. HFD-mediated NASH observed in WT was resulted from the induction of iNOS and NADPH oxidase, leading to increased nitroxidative stress, which then activated hepatic Kupffer cells with increased inflammation [117]. In addition, the coadministration of leptin with CCl4 to *ob/ob* mice significantly elevated nitroxidative stress compared to treatment with CCl4 alone [117]. Finally, the deletion of iNOS or p47 phox subunit of NADPH oxidase ameliorated the peroxynitrite formation and protein nitration [117], suggesting an important role of protein nitration, at least partially, in the leptin-mediated activation of Kupffer cells. Further, metal iron was also found to increase liver injury in diabetic rats, partially, through nitration of glucokinase accompanied with decreased levels of its expression and activity, shedding some new lights on the role of iron in the development of hepatotoxicity in diabetes mellitus [118]. Peroxynitrite and protein nitration were also reported to play a central role in the suppression of the mitochondrial respiratory chain activity, leading to mitochondrial dysfunction in a NASH model using *ob/ob* mice while these effects were ameliorated by melatonin administration [119]. The source of NO seems to be important in determining the outcome of the increased or decreased NO levels. For instance, in a HFD-mediated NAFLD rat model, simvastatin was found to protect against the development of HFD-induced liver fibrosis via differentially regulating NOS isozymes, where eNOS was found to be elevated while iNOS was decreased [112]. These results suggest that eNOS seems to be protective against NAFLD, while iNOS seems to promote NAFLD [112]. Protein nitration was also reported to play an important role in the development of insulin resistance in NAFLD in WT mice infused for 6 h with a 20% intralipid emulsion [120]. Further, iNOS induction and consequent Tyr-nitration of key insulin signaling proteins such as AKT, insulin receptor-β (IRβ), insulin receptor substrate-1 (IRS-1), and insulin receptor substrate-2 (IRS-2) [120] lead to the interference with Tyr phosphorylation, a signature feature of the insulin signaling pathways. In addition, there was increased serine phosphorylation, which leads to the inhibition of the hepatic insulin signaling pathway, especially when Tyr phosphorylation was inhibited [120]. These events were monitored with minimal changes in the basal levels of the insulin signaling molecules before and after lipid infusion [120]. Thus, a novel mechanism for hepatic insulin resistance caused by circulating lipids from HFD through protein nitration was suggested since these events were significantly ameliorated in *iNOS*(−/−) mice [120]. Furthermore, nitration of IRS-1 and IRS-2 proteins caused decreased expression of both proteins in obese diabetic rats or when hepa1c1c7 and HepG2 hepatoma cells were exposed to NO donors such as GSNO and SIN-1 in a time- and dose-dependent manner. In contrast, their expressed levels were increased after the hepatoma cells or obese diabetic rats were treated with an iNOS inhibitor, N6-(1-iminoethyl)-L-lysine (L-NIL) compared to their control [44]. The decreased protein levels of IRS-1 and IRS-2 following their nitration in these models of NAFLD might be due to their ubiquitin-dependent degradation, as discussed previously [53, 120]. Taken together, the outcome of these effects would depend not only on the extent of inactivation and/or loss of the nitrated proteins but also on the cellular ability to overcome the loss of these particular nitrated proteins by various cell defense mechanisms. Thus, it is reasonable to conclude from the two aforementioned

studies [44, 120] on HFD-mediated hepatic insulin resistance where insulin signaling molecules could be by nitrated without change in their protein levels probably occurring at early stages of the disease process (i.e., following a short-term exposure). Alternatively, insulin resistance could also result from the decreased levels of nitrated insulin signaling molecules via proteolytic degradation, usually observed at later time points following exposure to persistent insults.

Based on the aforementioned reports, future studies on the functional implications of protein nitration should consider the temporal evaluation of protein nitration and the remaining levels of nitrated proteins, since the nitration process is extremely dynamic and that the levels of nitrated proteins could vary dramatically over the course of acute or chronic hepatic insults. This is particularly important since there are many reports suggesting that protein nitration is not chemically stable, as previously believed. By using a reducing agent sodium dithionite as described previously [121], we showed that most of the nitrated cytosolic proteins, determined by immunoblot analysis following 2D gel electrophoresis, completely disappeared in APAP-exposed mouse livers [40]. The disappearance of nitrated protein spots was due to the reduction of nitro-Tyr to amino-Tyr, which was no longer recognized by the antibody to 3-NT. The reduction of nitro-Tyr to amino-Tyr was also reported by a pure chemical reaction from Fe3+-containing heme as in hemoglobin and myoglobin in the presence of a reducing agent [122]. Similar reaction has been also reported under physiological conditions where nitrated deoxynucleo bases were reduced to their amino analogues [123].

One might think that the decreased levels of nitrated proteins over time only result from their proteolytic degradation, as previously discussed; however, this is not necessarily true. For instance, in the presence of protease inhibitors to block proteasomal degradation of nitrated proteins, nitrated BSA disappeared when incubated with the homogenates of spleen or lung tissues, but not with the homogenates of rat liver or kidney, suggesting that different isoforms of denitrase might exist in a tissue-specific manner [124]. Protein with denitrase activity has been detected in a variety of tissues including the liver, heart, lung, brain, spleen, and kidney [31, 125, 126]. Denitrase activity has been suggested to be present in both aqueous and membrane phases in the cells [31]. Indeed, denitration reaction via denitrase was observed with nitrated Histone H1.2 [125], glutamine synthetase [127], calmodulin [128], L-type Ca^{2+} channel [129], cyclooxygenase [31], and so forth. Although constitutively active denitrase was present in many tissues and partially purified using the nitrated cyclooxygenase as a substrate [31, and references therein], the biochemical and regulatory properties of denitrase enzyme still need additional investigations. Collectively, data from these reports suggested that denitration may serve as an adaptive mechanism by which cells can repair damaged proteins. In addition, the nitration and denitration process can be of particular importance for cell signaling-related events. For a molecule to act as a signal, nitration-denitration has to be a reversible process. Indeed, nitrated cytochrome c was suggested to act as a signal molecule, as shown in cytochrome c overexpressing HeLa cells exposed to peroxynitrite with the spontaneous translocation of nitrated cytochrome c from mitochondria to cytosol and nucleus [130]. It is also very important to temporally study the nitric oxide bioavailability through the course of NAFLD. For example, in a mouse model of NAFLD fed a HFD for 8 and 16 weeks, NO contents were initially increased causing mitochondrial damage with mitochondrial protein alterations but decreased at later stages of the NAFLD [131]. The authors suggested that decreased NO might be involved in the progression of NAFLD to NASH. Again the decreased NO levels at later stages seem to be due to increased levels of arginase-1, depleting the substrate (i.e., L-arginine) of NOS and decreased levels of activated eNOS-PSer1177, which is protective against disease progression [112, 131]. However, in a clinical study using patients with different grades of liver cirrhosis, a positive correlation between the levels of nitrated proteins in plasma, platelets, and liver tissues and the severity of liver cirrhosis was found [132]. Thus, based on the results from previous reports [124, 132] and others, it is still unclear whether protein nitration can directly play a role in the initiation and/or development of NASH progressed from NAFLD. NAFLD is not only induced by pathological conditions such as obesity and diabetes but also by various diets such as HFD, fructose, and (methionine- and) choline-deficient diet [133–136]. Collectively these reports suggest a potential role of nitrated proteins in mediating NAFLD through various mechanisms (Figure 1).

2.1.3. Acute Liver Injury Caused by Drugs and Xenobiotics. There are many enzymes that are expressed in the liver and other extrahepatic tissues while their activities are compromised by Tyr-nitration following exposure to certain drugs (e.g., APAP, zidovudine (AZT), MDMA, tamoxifen, amiodarone, and so forth) or other toxic substances including lipopolysaccharide (LPS). These agents can provoke acute liver injury with or without fat accumulation probably through increased nitroxidative stress-mediated inhibition of the enzymes involved in the fatty acid oxidation pathway (i.e., mitochondrial β-oxidation) [38, 137, 138]. If an enzyme, essential for the energy production or detoxification of certain metabolites, is inhibited under elevated nitroxidative stress, this inhibition is likely to exert deleterious effects on the liver function and/or hepatic disease progression. For instance, mitochondrial carbamoyl phosphate synthetase 1 (CPS1), which is responsible for the detoxification of excess ammonia, was found to be inactivated when it is nitrated at Tyr1450 [33]. This decline in CPS1 activity may result in hyperammonemia and subsequent mitochondrial dysfunction in in vivo situations, contributing to liver damage and possibly injury to the brain. LPS dramatically increased protein nitration along with other parameters of oxidative stress such as lipid peroxidation in the livers of peroxisome proliferator-activated receptor-α (PPARα)(−/−) than in WT, which also exhibited significantly increased nitration compared to its untreated control, leading to elevated hepatic toxicity in *PPARα*(−/−) than WT [139]. Mitochondrial dysfunction was observed in LPS-exposed *PPARα*(−/−) mice, as evidenced by the inhibition of complex I and ATP synthase

activity, both of which were reported to lose their activities by nitration [36, 139]. Further, LPS exposure decreased the catalytic activity and expressed amount of hepatic mitochondrial glutamine synthetase (GS) in rat liver through nitration at multiple Tyr residues including the active site Tyr160 [34], accompanied by inactivation. Since GS is also responsible for ammonia elimination, a compromise in its activity and function likely contributes to sepsis-induced development of hyperammonemia in patients suffering from hepatic cirrhosis [34]. The disturbance in ammonia metabolism may not only affect the liver but also the brain, leading to a condition known as the hepatic encephalopathy.

Mitochondrial ATP synthase is essential for providing cellular energy (i.e., ATP) for proper maintenance and survival of all living cells. If ATP synthase (mitochondrial complex V) is inhibited, this leads to depletion of an essential energy source, thus contributing to acute necrotic liver injury. We showed that the activity of ATP synthase was significantly inhibited in mice subjected to I-R injury [140], in MDMA-exposed rats [38], in LPS-exposed *Ppara*(−/−) mice [139], and in mice exposed to a toxic dose of APAP [35]. In these studies, we confirmed its nitration by immunoblot analysis with the anti-3-NT antibody following the immunoprecipitation of ATP synthase. It is noteworthy to mention that, in all of these models, the protein levels of ATP synthase seemed unchanged in response to any of those treatments, suggesting that the inhibition of its activity was mainly due to nitration-mediated inactivation.

Several hepatic enzymes involved in cellular defense were reported to be nitrated and their activities inhibited in livers and other tissues or cultured cells after exposure to toxic insults. APAP can be considered a prime example of the role of protein nitration in drug-induced liver injury (DILI). It has been shown that nitro-Tyr protein adducts formation correlated very well with the areas of hepatic necrosis [141]. The direct evidence of the critical role of peroxynitrite in APAP-mediating hepatic injury has also been provided by the seminal work of Knight et al. [142]. This study showed that the protective effects of GSH, either cotreated with APAP or administered at different intervals up to 2.25 h post-APAP treatment, were resulted from the restoration of cellular GSH levels and the increased efficiency of scavenging peroxynitrite [142]. Similar results were also obtained in glutathione-peroxidase deficient animals [142]. In addition, Cover et al. [143] showed for the first time that APAP can selectively promote mitochondrial protein nitration. This study also provided evidence that peroxynitrite can cause mitochondrial DNA damage directly and/or indirectly via the mitochondrial damage pathway [143]. The events for APAP-induced protein nitration and liver injury seem dependent on the presence of CYP2E1, which is one of the major enzymes involved in the metabolism of APAP [40, and references therein]. Furthermore, in a comprehensive study using a toxic dose of APAP, nitration of cytosolic Cu/Zn-dependent superoxide dismutase (SOD1) eventually led to ubiquitin-mediated degradation and significant inhibition of its activity and protein levels in WT but not in the corresponding *Cyp2e1*(−/−) mice, as evidenced by immunoprecipitation using the anti-SOD1 antibody followed by immunoblot analysis with either anti-3-NT or antiubiquitin antibody. These results suggest a role of CYP2E1 in protein nitration and subsequent degradation [40]. Further, several reports showed that when isolated cells or proteins were exposed to peroxynitrite, the induction of proteolytic degradation was observed [54, 55, 144, 145]. The proteolytic degradation of bovine SOD1 was also increased by the 20S/26S proteasome when Tyr108 was nitrated [53]. Taken together, protein nitration can contribute to APAP-mediated toxicity via nitration-mediated inactivation and/or proteasomal degradation of essential proteins in antioxidant defense. The inhibition of antioxidant mitochondrial SOD2 also plays a critical role in promoting APAP-induced hepatotoxicity due to its nitration and subsequent inactivation, hampering cell's ability to defend itself against increased nitroxidative stress [146]. In addition, nitration of cytosolic glutathione reductase (GR) at Tyr106 and Tyr114 decreases GR binding to its substrate oxidized glutathione (GSH) and thus markedly decreased its activity with increased amounts of oxidized GSH [42, 43]. Although this reaction was observed in an in vitro system, it is likely that, in case of increased RNS, hepatic GR may become one of the enzymes that can be also inhibited through nitration. Persistently high levels of oxidized GSH may lead to a vicious cycle of oxidative stress development and sensitize the target tissue to an additional insult. All these results suggest that protein nitration also plays an important role, at least partially, in liver injury caused by acute, subchronic, and chronic exposure to a variety of drugs and hepatotoxic substances. It should be noted however that removal of nitrated proteins either via ubiquitin-dependent protein degradation [40, 53] or autophagy-dependent clearance of damaged mitochondria [147] can also be considered a hepatoprotective pathway. Based on these studies, it is likely that the ultimate hepatic effects of a toxic substance could be decided by the delicate balance between the occurrence and removal of nitrated proteins.

2.2. Activation of Nitrated Proteins and Functional Consequences. Another consequence of protein nitration is a gain of normal protein functions that can either be beneficial or harmful. For example, nitration at Tyr33 or Tyr56 of the 90-kDa heat-shock protein (Hsp90) is likely to convert the Hsp90 into a toxic protein through a gain of function. Using an antibody that recognizes the nitrated Hsp90 [48], nitrated Hsp90 immunoreactivity was detected in motor neurons of patients suffering from amyotrophic lateral sclerosis (ALS), in an animal model of ALS, and in an experimental model of spinal cord injury [48]. The authors concluded that nitration of a single important protein Hsp90 can initiate cell death while this protein can also become a potential target for therapeutic intervention [48]. This observation is also of particular interest in understanding the role of CYP2E1 and the mechanism of acute and chronic liver injuries where elevated CYP2E1 is known to play an important role in mediating these injuries. Hsp90 was found to interact with membrane-bound CYP2E1 forming a binary complex, which then transfers the membrane-bound CYP2E1 to the proteasome complex for its degradation [148]. Ethanol was found to compromise this interaction between Hsp90 and CYP2E1,

resulting in increased levels of CYP2E1 protein expression, which can damage the liver cells through its nitroxidative stress-mediated events [148]. These results are consistent with the inhibition of Hsp90 by geldanamycin, which increased CYP2E1-mediated toxicity in HepG2 hepatoma cells [149, 150]. Since both acute binge and chronic ethanol administration also promote hepatic protein nitration, it would be interesting to evaluate whether Hsp90 is nitrated in these alcohol-exposure models and whether this nitration leads to hepatic injury directly or indirectly through interference of binding between nitrated Hsp90 and CYP2E1, leading to its elevation.

The antioxidant glutathione *S*-transferase 1 (GST-1) was reported to be nitrated at Tyr92 and Tyr152. However, nitration at Tyr92 was shown to be critical in functional change as another example of gain in protein function. This conclusion was evidenced by site directed mutagenesis of each Tyr residue of GST-1, overexpression of each GST-1 mutant in LLC-PK1 cells, and then exposure to peroxynitrite followed by monitoring its activity [41]. The activation of GST-1 nitrated at Tyr92 would improve the antioxidant defense. Tyr nitration was also found to be essential for initiating the seeding process followed by protein aggregation in fibrin for blood clot formation [151] and for aggregation of α-synuclein, a component of Lewy bodies in many neurological diseases [54, 55, 152]. In addition, Tyr nitration of the catalytic subunit of protein phosphatase type 2A (PP2A) seems important in reactivating PP2A activity and promoting LPS and interferon-γ induced microvascular endothelial barrier dysfunction with increased albumin permeability. This gain of function by nitrated PP2A was resulted from interference with Tyr phosphorylation of the catalytic subunit, which subsequently suppresses PP2A activity [49]. All these examples, especially the antioxidant GST-1 and PP2A, with increased activities and functions through protein nitration, are of interest and one needs to take extra cautions against premature conclusions that protein nitration is considered only as a pathological risk factor without proper evaluation of the enzyme activity and functional consequence.

2.3. Induction of Immunogenicity by Nitrated Proteins and Potential Implications in Various Liver Diseases. Another consequence of protein nitration is the induction of immunogenicity and immunomodulation. The body system tolerates its endogenous proteins without eliciting an immune response through immunological tolerance. However, once protein secondary structures are modified via various PTMs, the body immune system might not tolerate the modified proteins and thus immune response might ensue. This immune activation may be due to the structural changes following protein nitration or any other PTMs such as oxidation, carbonylation, phosphorylation, and glycation including AGE, acetylation, *S*-nitrosylation, glutathionylation, and transglutamination. All these PTMs may contribute to the exposure of a new antigenic epitope, leading to stimulation of a cascade of immune response that starts with the activation of B and/or T cells [153]. This immune reaction can be beneficial when under normal conditions where it clears out a harmful complex or can be hazardous if its level becomes abnormal and sustained through a vicious cycle. Thus the aggregated nitrated proteins in various tissues, including the liver, as seen in inflammation, for example, [154], can initiate or exaggerate an inflammatory reaction that might even become chronic, via the stimulation of the immune system against its own proteins, known as an autoimmune response [155]. Indeed, elevated levels of anti-3NT antibodies were reported in the plasma of patients with post-traumatic acute lung injury [156] and in the synovial fluids of patients with rheumatoid arthritis and osteoarthritis as well as in the serum of patients with the autoimmune disease systemic lupus erythematosus [153]. However, Tyr nitration does not always invoke an immune response; instead it might also inhibit the immune response when it seems necessary in some cases. For instance, nitration of T cell receptor—CD8 complex—compromises its ability to bind to the major histocompatibility complex dimers, leading to tolerance of T cells to cancer cells and hence the survival of cancer cells [157, 158]. Thus protein nitration might be involved in immunomodulation, leading to the development of autoimmune diseases and even cancer progression. Since hepatic protein nitration is remarkably elevated in various chronic diseases including inflammatory diseases, aging, and cancers, it would be valuable to carefully evaluate the role of protein nitration in pathophysiology of these diseases. Alternatively, protein nitration can regulate immune function by interfering with Tyr phosphorylation and concurrent activation of various signal transducer and activators of transcription (STAT) proteins, as reported [159, 160].

2.4. Lack of Functional Consequences of Nitrated Proteins. Finally protein nitration might not have any significant effect on protein activity, expression, and consequently function, as reported with Tyr nitration of transferrin and α1-antichymotrypsin in acute respiratory distress syndrome [161]. It is vital to keep in mind, however, that many studies in the literature with regard to the Tyr nitration or denitration of certain proteins and subsequent functional changes were based on in vitro reactions using high concentrations of peroxynitrite or other nitration-inducing substances and in an environment lacking the full host defense system including various antioxidants present in living organisms. Thus, the nitrative effect of any agent on protein function also needs to be validated in in vivo systems. It is still challenging to determine whether catalytic inhibition or degradation of a particular protein is solely resulted from its Tyr nitration as there are many other forms of PTM that can also inhibit its activity.

Table 1 summarizes some nitrated liver proteins with functional consequences with the reported or suggested consequences.

3. Protein Nitration in Mitochondria and Potential Consequences in Various Liver Diseases

Protein Tyr nitration can occur in different cell compartments and play a critical role in hepatic injury. However,

based on the aforementioned studies and alterations of key mitochondrial proteins involved in fat and ammonia metabolism, energy supply, antioxidant defense, and so forth (listed in Table 1 and references therein), mitochondrial functional status can become a very important factor in the initiation and/or progression of liver diseases. In addition, NO-dependent inhibition of the mitochondrial respiration was reported in both AFLD and NAFLD [16, 73, 162]. However, most of the reported hepatic studies, with a few exceptions, indicated the pathological role of nitrated proteins in mediating hepatic disease process, relying mainly on the correlation between protein nitration and hepatic injury without in-depth analysis of functional alterations in nitrated mitochondria proteins. Therefore, we briefly describe the formation of protein Tyr nitration and functional consequences that might explain the role of protein nitration in promoting many forms of liver diseases including AFLD and NAFLD.

Under normal physiological conditions, approximately 1%-2% of oxygen leaks out as ROS from the mitochondrial electron transport chain (ETC) [163]. ROS can either combine with protons to produce water [164, 165] or can be handled with mitochondrial antioxidant defense enzymes where these radicals are dismutated by SOD2 and detoxified by glutathione peroxidase (GPX) [165]. Alternatively, these ROS can be involved in various cellular signaling pathways [166]. However, damaged mitochondria in various pathological conditions or exposure to toxic agents such as alcohol, HFD, LPS, abused substances (e.g., MDMA), or other therapeutic drugs (e.g., APAP and AZT) [137, 164, 167, 168], greater amounts of ROS are leaked out from the mitochondrial ETC, possibly at the sites of Complex I and Complex III, as suggested by alcohol-subjected hepatocytes [169]. RNS including NO radicals can also be detected in the mitochondria after NO is produced in cytosol by the NOS isozymes since NO can readily cross the mitochondrial membranes. In addition, mitochondrial RNS can be produced by mitochondria NOS [170]. Thus the excess of ROS and RNS radicals in the mitochondria can lead to the formation of peroxynitrite and hence increased mitochondrial protein Tyr nitration. Mitochondrial protein nitration can also be mediated through the oxidation of Tyr and nitrite via a peroxidase-catalyzed reaction [171, 172]. Mitochondria, which are the major source of cellular ROS, also have low levels of antioxidants such as reduced glutathione (GSH), compared to that in cytosol [173], rendering mitochondria a vulnerable target for nitrative damage. It has also been suggested that rat liver mitochondrial protein turn-over rates were dramatically decreased from days to hours upon exposure to nitrating conditions due to proteolytic degradation [46, 174–176]. Further, mitochondria under nitroxidative stress in animal models or human disease specimens show abnormal and irregular shapes and decreased functions [177, 178]. Nitration of many mitochondrial proteins can further deteriorate mitochondrial functions through inactivation of some critical proteins for cell maintenance and survival. For instance, suppressed activities of antioxidant enzymes such as SOD2, GPX, and GR would lead to decreased antioxidant cell defense, while suppressed ATP synthase may result in decreased ATP production with compromised cellular ability to perform normal functions and increased necrosis. Furthermore, inactivation of thiolase likely leads to the inhibition of the β-oxidation of fatty acids with increased hepatic fat accumulation or inefficient supply of an alternative energy (e.g., ketone bodies produced from fat degradation) when glucose supply is decreased during fasting or not efficiently utilized in many disease states. Suppression of mitochondrial aldehyde dehydrogenase 2 (ALDH2), involved in the metabolism of reactive acetaldehyde and 4-hydroxynonenal, would lead to accumulation of reactive aldehydes including lipid peroxidation products ([179], Table 1 and references therein).

Mitochondrial functions can also be compromised via deletion and/or mutation through oxidative/nitrative modifications of mitochondrial DNA, which is important as they encode 13 polypeptides that are all subunits of the 4 mitochondrial ETC proteins (i.e., Complexes I, III, IV, and V) [168, 180]. Mitochondrial DNA is also extremely sensitive to nitroxidative damage due to its location within the cell close to the inner mitochondrial membrane, and the relatively low levels of protective antioxidant enzymes, histone proteins, or polyamines and DNA repair enzymes in mitochondria compared to other subcellular organelles including nuclei ([138, 181] and references therein). In addition, it has been suggested that the rate of mutation in mitochondrial DNA is 10-fold higher than that in the nuclear DNA [182]. Taken together, oxidative damage and/or deletion of mitochondrial DNA [183, 184] may lead to reduced expression and function of mitochondrial ETC proteins, contributing to greater ROS production, as shown in alcohol-exposed rats [70]. Lipid peroxides can further alter the cell membrane functions and promote fibrosis through activation of stellate cells with elevated production of collagen and proinflammatory cytokines/chemokines that promote recruitment of neutrophils and activation of hepatic macrophage Kupffer cells. All these events contribute to profound deleterious effects on mitochondrial functions with increased steatosis, apoptosis, and necrosis. In addition, activation of autophagy and consequent removal of damaged mitochondria by ethanol [185] or APAP [147] have been suggested to be an adaptive and protective pathway against hepatic damage by both substances. The removal of damaged mitochondria under elevated nitroxidative stress may also be a mechanism by which cells can get rid of the nitrated proteins to protect themselves. Collectively, these studies and mechanisms support the notion that oxidative/nitrative stress produces mitochondrial dysfunction that can play a major role in stimulating the damage in the various hepatic diseases including AFLD and NAFLD and also extrahepatic tissue injury including brain diseases [182, 186–188]. Figure 1 summarizes the different pathways that nitrative stress might cause mitochondrial dysfunction, contributing to tissue injury including AFLD and NAFLD.

4. Detection Methods of Nitrated Proteins and Challenges

Nitrated proteins should be isolated or enriched before they are subjected to gel electrophoresis separation and protein

digestion followed by final MS analysis to identify proteins and possibly Tyr-nitrated peptides. Several methods have been reported to detect Tyr nitration including, but not limited to: (1) two-dimensional gel electrophoresis followed by immunoblot analysis with specific anti-3-NT antibody [189], (2) immunoprecipitation with the specific antibody [47, 190] or immunoaffinity chromatography using immobilized specific anti-3-NT antibody on Sepharose followed by capturing nitrated proteins [191], (3) solution isoelectric focusing, and (4) redox proteomic approach via conversion of nitro-Tyr to amino-Tyr followed by its labeling with biotin or dansyl chloride [192] to isolate nitrated proteins [193, 194]. Recent reviews summarized standard methods for the identification and quantification of proteins nitrated at the Tyr residues through purification of nitrated proteins and in-gel or in-solution trypsin digestion followed by gas chromatography (GC)—or liquid chromatography (LC)—tandem MS analyses [51, 195]. The identification of peptides that originate from nitrated proteins can be performed by using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS [51, 195–197], while identification of specific nitrated Tyr residues can be performed by LC-electrospray ionization (ESI)-MS/MS [198]. Despite the fact that all techniques have merits, there are many short outcomes that can be improved especially when it comes to the number of identified proteins and the ability to capture proteins that is not highly expressed or the capturing of nonspecific proteins. Many excellent reviews have been already reported with more details about nitrated protein isolation and characterization protocols and the advantages and disadvantages of each method ([51, 199, 200] and references within).

Our laboratory recently developed a simple approach to efficiently identify and characterize protein nitration and functional consequences. Although the methods of immunoaffinity chromatography were described before [191, 201], the recovery of the nitrated proteins was low. An attempt to improve the method was undertaken by increasing the incubation time of the sample with the immunoaffinity resin to 19 h and by also improving the quality and specificity of captured proteins by using more stringent washing protocol [202]. However, the incubation time in this protocol seems too long and not very practical in purifying sufficient amounts of nitrated proteins for MS analysis especially when nitrated proteins are expressed in very low quantities.

After preparing tissue homogenates in buffer preequilibrated with nitrogen gas for 1 h to remove dissolved oxygen, the levels of Tyr-nitrated proteins in the target sample compared to control should be determined by immunoblot analysis with anti-3-NT antibody. Once different levels of protein Tyr-nitration between the target and control samples are confirmed, whole homogenates should be further fractionated to prepare lysates from specific subcellular organelles (e.g., mitochondria versus cytosol). Nitrated proteins in mitochondrial extracts from treated and control samples, for instance, are then affinity purified by using the commercially available agarose beads coupled with anti-3-NT antibody, as similar to the method [203]. The affinity-purification steps should be repeated several times to collect sufficient amounts of nitrated proteins. The affinity-purified proteins can be concentrated using mini-spin-column concentrators (Millipore). Small aliquots of purified nitrated samples from control and treated or disease group can be separated on 1D gels and stained with silver to confirm the differences before MS analysis for protein identification. Nitration of some selected proteins identified by MS analysis could be individually confirmed by using immunoprecipitation with the specific antibody against each protein followed by immunoblot with anti-3-NT antibody. In addition, the functional consequence of protein nitration should be determined by measuring the catalytic activity of each target protein [35]. It would be ideal to further determine the nitrated Tyr residue(s) and function by site-directed mutagenesis followed by overexpression and activity measurement. Finally, it is always desirable to determine the causal relationship between Tyr-nitration detected earlier and the full-blown pathological states or tissue injury, usually observed at later time points, following exposure to the toxic substances, as described [35, 140]. Using this method of immunoaffinity purification, we were able to identify more than 30 and 70 nitrated cytosolic and mitochondrial proteins, respectively, in APAP-treated mouse liver. Detection and functional consequences of nitration in five selected mitochondrial proteins (Figure 2 and Table 1) were confirmed via immunoprecipitation followed by immunoblot analysis with anti-3-NT antibody and activity measurements in the absence or presence of an antioxidant, which fully prevented APAP-mediated protein nitration and hepatic damage [35]. Tyr-nitrated peptides of many proteins including SOD2 in APAP-exposed mice could not be identified by the MS analysis. However, nitration of four or five Tyr residues including the critical Tyr34 and inactivation was further confirmed by the MS analysis and activity measurement, respectively, after recombinant SOD2 protein was incubated with a nitrating agent tetranitromethane for 20 min [35].

Based on our recent results, we believe that the immunoaffinity purification followed by MS analysis may represent the best approach among all the aforementioned methods for identifying Tyr nitrated proteins. This approach can be also used to purify sufficient amounts of nitrated proteins for studying the role of protein nitration in various disease models, different organs, and/or different subcellular organelles such as nucleus and endoplasmic reticulum where identification of nitrated proteins can be challenging due to the low yield in isolation of nitrated proteins. However, there are still many challenges with purification and functional characterization of nitrated proteins. Although we and other laboratories reported many nitrated proteins [35, 47, 179, 189–200], and Table 1 it is conceivable that the actual nitrated proteins should be far greater than those we identified in APAP-exposed WT mice. Some nitrated proteins are transiently expressed while the amounts of other nitrated proteins could be decreased possibly through proteolytic degradation upon nitration, as previously mentioned. Therefore, it is difficult to capture and characterize all nitrated proteins analyzed at one time point. By reviewing the literature and based on our own data, we acknowledge that identification of certain nitrated transcription factors, insulin receptors, nuclear receptors, and signaling molecules, for example,

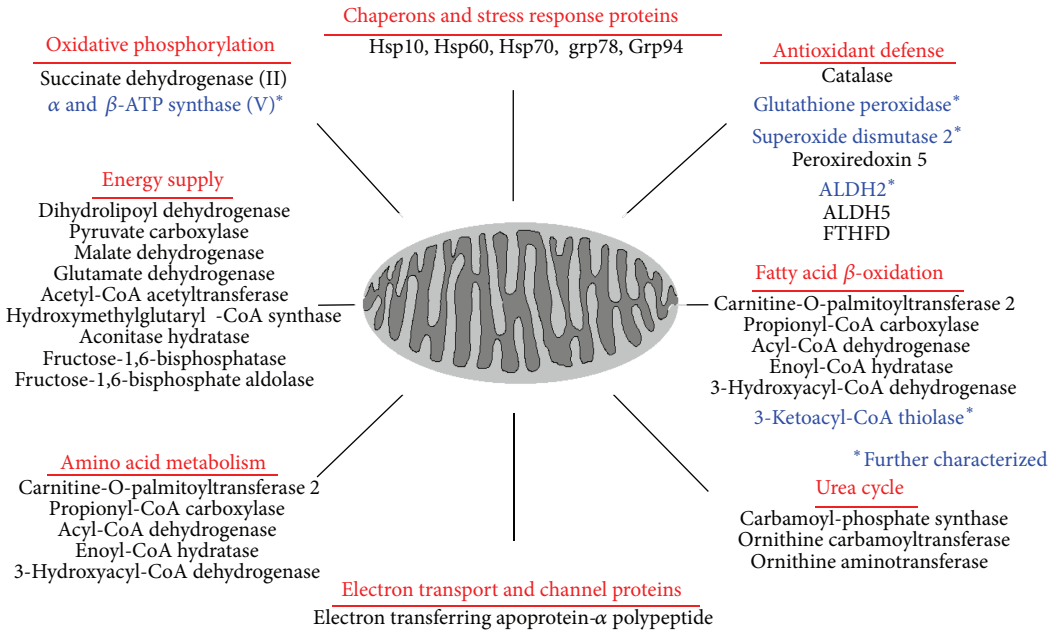

Figure 2: Summary of nitrated mitochondrial proteins in acetaminophen (APAP)-exposed mice. Some recently-identified nitrated mitochondrial proteins in APAP-exposed mouse livers are summarized with respect to the function of each protein identified by mass spectrometry. *Five selected proteins (marked in bold blue) were further characterized for the reversible changes and enzyme activities after APAP exposure in the absence or presence of NAC co-treatment, as described in and adapted from [35].

could be particularly difficult even after repeated purification steps due to extremely low levels of expression. However, individual characterizations of these factors could be of extreme importance to understand the mechanisms of their well-documented alterations in various disease conditions including AFLD and NAFLD. However, this technical problem could be overcome by immunoprecipitation of the individual protein of interest followed by immunoblot with the specific anti-3-NT antibody and measurement of a functional activity in the absence or presence of a peroxynitrite scavenger. Alternatively, the nitrated proteins of interest could be purified to examine the consequence of their alteration under nitration-inducing conditions. One promising approach could be to employ an in vitro system by using isolated mitochondrial proteins exposed to a nitrating agent or incubated with recombinant NOS. This in vitro method is likely to allow identification of many nitrated mitochondrial proteins. However, the level of peroxynitrite produced by this protocol seems far greater than that generated in in vivo conditions and the in vitro results cannot be used to correlate with samples from animal models or human specimens without extra precaution [46]. Thus there are still many challenges that need to be addressed in future research.

5. Translational Research Using Nitration Proteomics Approaches to Evaluate Beneficial Agents to Prevent or Treat Hepatic and Other Tissue Injuries

Upon understanding the potential mechanism(s) of nitroxidative stress-related liver diseases, the next logical extension would be translational application to identify beneficial agents to either prevent or treat the disease progression. The relative levels of nitrated proteins between controls versus disease samples must be evaluated prior to conducting translational research with some protective agents administered before and after or during disease progression in experimental models. We recently reported this kind of translational application by first characterizing the mechanism of APAP-induced tissue injury followed by efficacy evaluation of an agent against liver damage [35, 40, 78]. Firstly, we demonstrated the importance of CYP2E1 and protein nitration in APAP-mediated hepatotoxicity by comparing the phenotypic changes in WT and *CYP2e1*(−/−) mice [40]. In this study, we also showed that one of the mechanisms of nitration-mediated tissue injury was suppression (inactivation) of vital proteins such as SOD1 through ubiquitination and subsequent degradation [35, 40, 78]. These results are consistent with the reports by other scientists [204–207]. In continuation, we then immunoaffinity purified and determined identities of many nitrated proteins in both mitochondria and cytosol at earlier time point (e.g., 2 h after APAP treatment). Five critical enzymes SOD2, GPX, ATP synthase, ALDH2, and thiolase were selected for further characterization with respect to their nitration and activity changes at 2 h post-APAP treatment in the absence or presence of NAC. The results revealed that NAC treatment restored APAP-induced changes in transaminase activities, liver histology, and protein nitration to normal levels. NAC also reversed the inactivation of the five enzymes via Tyr nitration, as evidenced by immunoprecipitation with a specific antibody to each protein followed by immunoblot with anti-3-NT antibody. All these results demonstrate the causal roles of protein nitration in

promoting full-blown liver damage usually observed at later time points (e.g., 16 or 24 h after APAP treatment) [35]. The combined results of the two studies [35, 40] about the critical role of protein nitration in APAP-induced liver damage and beneficial effect of NAC are consistent with the reports by other laboratories [208, 209]. Thus, our approaches not only allow the characterization of many nitrated mitochondrial proteins but also provide an opportunity for translational research in evaluating the benefits and mechanisms by which NAC protects from APAP-induced liver injury.

This kind of approach can also be applied to future translational studies on various experimental models or human disease specimens where protein nitration seems to play a key role in promoting disease development while the efficacies of beneficial agents can be evaluated, as exemplified in a model of I-R-related acute liver injury. In this study, the benefit of a peroxynitrite scavenger metalloporphyrin MnTMPyP against I-R-related mitochondrial dysfunction and acute hepatotoxicity was investigated [140]. MnTMPyP pretreatment markedly suppressed the I-R-related elevation of serum transaminase levels, histological damage, iNOS expression, and oxidative modifications of key mitochondrial proteins. MnTMPyP treatment also restored the activities of some essential mitochondria enzymes including ALDH2, thiolase, and ATP synthase inhibited under I-R condition. Based on our recent data [35], the activities of these mitochondrial enzymes in the I-R-mediated acute liver injury model could have been inhibited by protein nitration and then restored in the presence of a peroxynitrite scavenger. For this reason, the levels of nitrated proteins should be also evaluated in the future translational studies when testing the benefit of a protective agent.

Mitochondria are important for energy supply, antioxidant defense, apoptosis, and intermediary metabolism (including ammonia, urea, heme, and so forth) and fatty acid oxidation to provide an alternative energy source of ketone bodies when glucose supply is limited [210–212]. In this regard, it would be of particular importance to characterize the nitrated mitochondrial proteins to gain new mechanistic insights in mitochondrial dysfunction and disease progression in acute and chronic liver diseases in the absence or presence of a protective agent. In addition, the proteomics approach of characterizing nitrated proteins can be very useful in the evaluation of many antioxidants other than NAC such as *S*-adenosylmethionine [213], curcumin [214, 215], and green tea extracts, which decreased nitroxidative stress-mediated development of NAFLD in obese (*ob/ob*) mice [216]. Furthermore, this approach can be used in identifying new disease biomarkers in various organs and various disease stages in experimental models or clinical specimens. For instance, the levels of nitrated proteins in plasma, platelets, and liver tissue were positively correlated with the severity of hepatic cirrhosis [132] and diet-induced obesity [217]. In fact, an excellent review article for identifying potential biomarkers from its discovery to its application in clinical studies has been recently reported [218]. The initial stage is known as the discovery phase with identification of biomarker candidates. Various systems biology approaches including bioinformatics, genomics, proteomics, and metabolomic analysis should be used simultaneously to identify potential biomarkers in experimental animal models or human specimens with a specific disease of interest. The potential biomarkers with relative long half-lives and stabilities have to be evaluated or verified in a large number of patient samples with certain disease states for clinical validation purpose.

6. Concluding Remarks

Many reports suggest that PTMs, including Tyr nitration, seem to play an important role, at least partially, in various pathophysiological conditions including AFLD and NAFLD. The causal pathologic role of nitrated proteins, however, was questioned due to their relatively rapid turnover, degradation, and removal of damaged mitochondria by autophagy. It can be argued that the persistence of the insult-mediated protein nitration over extended periods of time and/or consequent imbalance between protein nitration and turnover can alter protein function. In addition, some of the nitrated proteins may disrupt normal signaling pathways (e.g., insulin signaling pathway), contributing to initiation of a cascade of deleterious events. Alternatively, other nitrated proteins may stimulate hepatic macrophage Kupffer cells with elevated cytokines and chemokines, which promote infiltration of neutrophils and inflammatory tissue injury. When these signaling pathways are initiated, even the removal of nitrative stress or nitrated proteins may not stop the ongoing destructive cascades. These events may explain the fact that certain diseases, albeit depending on disease stages, may not be fully treated or reversed even with the FDA-approved therapeutic drugs.

Nitrated proteins may not only play a role in mediating acute liver injury including DILI but also serve as a biomarker for some hepatic diseases. Due to the many forms of protein PTM, it is still challenging to dissect the unique role of nitrated proteins in promoting certain disease states. Further, many studies relied on positive correlation between protein nitration and injury development without detailed mechanistic studies. Thus, to investigate the role of nitrated proteins, a systematic and comprehensive approach should include identification, biochemical characterization, evaluation of overall consequence, and potential prevention or reversal of nitrated proteins and subsequent tissue injuries in the absence or presence of beneficial agents. This systematic approach would serve as a platform for the development of therapeutic agents against AFLD and NAFLD. However, extra caution should be taken into consideration regarding the functional consequence of any nitrated protein since nitration can also activate certain proteins (e.g., microsomal GST-1), as discussed above. It is also important to use sensitive and advanced methods to cover most peptide areas of a target protein to avoid or minimize the potential overlook in identifying nitrated peptides or proteins.

Protein nitration seems to occur in many subcellular compartments including mitochondria. Because of low levels of glutathione and antioxidant enzymes in mitochondria than in cytosol, mitochondrial lipids, DNA, and proteins can be more susceptible to oxidative and nitrative modifications

under increased nitrative stress, leading to mitochondrial dysfunction and tissue injury (Figure 1). In fact, many studies dealt with experimental disease models and clinical specimens from patients showed that mitochondrial proteins are abundantly nitrated and/or oxidatively modified, often accompanied with altered functions. Thus, future studies should lead to better understanding of the underlying mechanism(s) of mitochondrial dysfunction through nitration of many additional mitochondrial proteins and their functional implications in disease development and progression. In addition, the occurrence of protein nitration in different liver cells including hepatocytes, Kupffer cells, endothelial cells, and hepatic stellate cells and their functional implications in various liver diseases are poorly understood although these areas should be characterized further. Furthermore, future translational research should also include the development of mitochondria-directed antioxidants or peroxynitrite scavengers to prevent protein nitration and ultimately treat AFLD, NAFLD, and DILI as well as various diseases in other organs.

Abbreviations

3-NT: 3-NitroTyr
AFLD: Alcoholic fatty liver disease
AGE: Advanced glycation end products
ALDH2: Aldehyde dehydrogenase-2
ALS: Amyotrophic lateral sclerosis
CPS-1: Carbamoyl phosphatesynthase-1
CYP2B6: Cytochrome P450 2B6
CYP2E1: Ethanol-inducible cytochrome P450 2E1
DILI: Drug induced liver injury
ER: Endoplasmic reticulum
ETC: Electron transport chain
GR: Glutathione reductase
GS: Glutamine synthetase
GSH: Glutathione
GPX: Glutathione peroxidase
GST-1: Glutathione *S*-transferase-1
HFD: High fat diet
Hsp90: The 90-kDa heat-shock protein
I-R: Ischemia-reperfusion
IRβ: Insulin receptor-β
IRS-1: Insulin receptor substrate
iNOS: Induced nitric oxide synthase
LC: Liquid chromatography
LPS: Lipopolysaccharide
MS: Mass spectrum
NAC: *N*-Acetyl cysteine
NAFLD: Nonalcoholic fatty liver disease
NO: Nitric oxide
PTM: Posttranslational modification
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
PP2A: Protein phosphatase type 2A
PPAR-α: Peroxisome proliferator-activated receptor-α
SOD1: Cu/ZN superoxide dismutase
SOD2: Mn superoxide dismutase
Thiolase: 3-Ketoacyl-CoA thiolase
Trp: Tryptophan
Tyr: Tyrosine.

Conflict of Interests

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

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Research Article

Nitric Oxide Synthetic Pathway in Patients with Microvascular Angina and Its Relations with Oxidative Stress

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A decreased nitric oxide (NO) bioavailability and an increased oxidative stress play a pivotal role in different cardiovascular pathologies. As red blood cells (RBCs) participate in NO formation in the bloodstream, the aim of this study was to outline the metabolic profile of L-arginine (Arg)/NO pathway and of oxidative stress status in RBCs and in plasma of patients with microvascular angina (MVA), investigating similarities and differences with respect to coronary artery disease (CAD) patients or healthy controls (Ctrl). Analytes involved in Arg/NO pathway and the ratio of oxidized and reduced forms of glutathione were measured by LC-MS/MS. The arginase and the NO synthase (NOS) expression were evaluated by immunofluorescence staining. RBCs from MVA patients show increased levels of NO synthesis inhibitors, parallel to that found in plasma, and a reduction of NO synthase expression. When summary scores were computed, both patient groups were associated with a positive oxidative score and a negative NO score, with the CAD group located in a more extreme position with respect to Ctrl. This finding points out to an impairment of the capacity of RBCs to produce NO in a pathological condition characterized mostly by alterations at the microvascular bed with no significant coronary stenosis.

1. Introduction

NO is an important signaling molecule involved in the maintenance of vascular function. It promotes several beneficial effects in the vasculature by inducing vasorelaxation, inhibition of leukocyte-endothelium adhesion, smooth muscle cells migration and proliferation, and platelet aggregation [1, 2]. A decreased NO bioavailability is well documented in several cardiovascular diseases, including hypertension, atherosclerosis, and ischemia-reperfusion injury. A reduction of circulating NO species (nitrite and nitrosylated compounds), which contribute to the total NO availability, is described in individuals with endothelial dysfunction. The decrease is correlated with increasing numbers of cardiovascular risk factors [3–5].

NO is synthesized by the enzymatic action of NO synthases (NOSs), catalyzing the oxidation of the amino acid L-arginine (Arg) to equimolar amounts of NO and L-citrulline (Cit), in the presence of oxygen and cofactors. Although synthesis and release of NO are related to the substrate bioavailability [6], other potential causes of NO deficiency in disease settings have been proposed. Among these, the high circulating levels of endogenous methylarginines, that is, symmetric, asymmetric dimethylarginine (SDMA, ADMA) and monomethylarginine (MMA), act as NO-synthesis inhibitors [7, 8]. In addition, oxidative stress plays a pivotal role in determining NO bioavailability by the oxidation of the cofactors/the enzymes involved in NO metabolism or by the direct inactivation of NO.

Endothelial cells are considered the major source of NO in the vasculature; however, it has been shown that also circulating cells may contribute to NO synthesis, that is, platelets, monocytes, and red blood cells (RBCs). RBCs express

functional NOS [9, 10], similar to the enzyme of endothelial cells [11], which serves as an intraluminal NO source and contributes to the regulation of systemic blood pressure [12]. In addition, the transporter for cationic amino acids [13] and all the enzymes involved in dimethylarginine metabolism (synthesis and catabolism) [14] have been identified in RBCs. Human RBCs also express the enzyme arginase that competes with NOS for their common substrate Arg to form L-ornithine (Orn) [15]. Two different isoforms of arginase are expressed in human [16] and, recently, it has been shown that arginase I plays an essential role in the control of RBC-NOS function and in the release of bioactive NO [17]. Indeed, in experimental models of atherosclerosis [18], myocardial ischemia [19], hypertension [20], and ageing [21], arginase activity has been reported to be upregulated at vascular level.

Microvascular angina (MVA) is a pathological condition characterized by the typical anginal pain, electrocardiographic (ECG) abnormalities at rest (ST-segment depression or T-wave inversion), all features that increase during exercise, in the presence of nonobstructed epicardial coronary arteries [22–24]. Even if the pathophysiology of MVA has not been disentangled yet, insulin resistance, abnormal autonomic control, enhanced sodium hydrogen exchange activity, abnormal cardiac sensitivity, and microvascular spasm have been proposed as potential causes [25]. In addition, increased concentrations of circulating C-reactive protein have been shown to correlate with vascular abnormalities in patients with MVA, suggesting a role of inflammation in this pathological condition [26].

Oxidative stress *per se*, either directly or through the reduction of NO bioavailability leading to an impairment of endothelium dependent vasodilation, has been involved in the pathophysiology of MVA [27, 28]. In particular, impaired endothelium-dependent vasodilatation of the coronary microvasculature [27] and its related impaired function, which limits coronary flow reserve [28–30], have been proposed to induce MVA syndrome.

Alterations in flow-mediated coronary dilation are a frequent finding in patients with MVA. In the microcirculation, blood flow is largely dependent on hemorheological properties, particularly RBC deformability, whose importance increases in capillaries compared with larger vessel [31]. A decreased RBC deformability has been shown in patients with CAD and diabetes mellitus [32] and it has been related to a decreased NO release [33]. In addition, due to the structural properties and blood flow in the microcirculatory bed, blood cells are in close contact with endothelium. As it has been shown that eNOS expression decreases in the microvasculature [34], it could be speculated that within capillaries RBC-NOS may play a more decisive role [35].

Moreover, it has been recently shown that in patients with cardiac syndrome X an increase of red cell distribution width (RDW), a measurement of size variability, and of erythrocytes, occurs [36]. Even if it has been reported that reduction of nitrate and nitrite, coupled to increases in ADMA and SDMA, occurs in plasma of MVA patients [37, 38], no information on the levels of the single components of the NO pathway in RBCs is available yet. Thus, in this study, we have characterized oxidative stress and the NO biosynthetic pathway in RBCs of MVA patients in comparison to patients with coronary artery disease (CAD) or healthy subjects (Ctrl).

2. Methods

2.1. Study Population. Patients with MVA ($n = 25$) characterized by stable effort angina or inducible ischaemia and reduction of the coronary flow reserve, documented by a positive stress test (at least 2.0 mm horizontal or downsloping ST-segment depression) or by a positive SPECT, despite the absence of angiographically documented coronary disease, were recruited. These patients were compared with angiographically documented CAD patients ($n = 22$) and with subjects deemed as healthy on the bases of the absence of clinical symptoms, the instrumental and laboratory examination (Ctrl = 20), and the negative stress test from a previously described cohort [10]. Exclusion criteria were considered as follows: a history of congestive heart failure, significant valvular diseases, hypertrophic cardiomyopathy, vasospastic angina, recent (<6 months) acute coronary syndrome, surgical or percutaneous revascularization, pacemaker dependency, and atrial fibrillation. Patients with renal insufficiency (serum creatinine concentration >1.4 mg/dL), hepatic disease, recent infection, recent major surgical interventions, immunological disorders, and chronic inflammatory or neoplastic diseases were also excluded. This observational study was carried out in accordance with the Declaration of Helsinki and approved by the local ethics research committee of Centro Cardiologico Monzino (number S1687/610). Written informed consent to participate was obtained from all subjects.

2.2. Blood Collection. EDTA-anticoagulated blood was drawn from the antecubital vein of subjects while fasting to obtain whole blood, plasma, and erythrocyte samples. After centrifugation (1,200 g for 10 min at 4°C), plasma was separated and aliquots were stored at −80°C until analyses. Aliquots of packed red cells were lysed by cold deionized water to obtain lysed RBCs and stored at −80°C until analyses.

2.3. Arg/NO Metabolic Pathway. We simultaneously measured Arg, ADMA, SDMA, MMA, Cit, and Orn by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [39]. The ratio Arg/(Orn + Cit), as index of global Arg availability [40, 41], and the ratio Orn/Cit, as indicator of the relative activity of arginase and NOS [19], were computed. All the determinations were performed both in plasma and in lysed RBCs.

2.4. Oxidative Stress. It was evaluated by the ratio between disulphide and reduced forms of glutathione (GSSG/GSH). GSH and GSSG were measured by LC-MS/MS method on whole blood, after proteins precipitation with trichloroacetic acid [42]. Levels of GSH and GSSG were expressed as μmol/g Hb.

2.5. RBC-NOS and Arginase Expression. The RBC-NOS expression was performed by immunofluorescence analysis

in a subgroup of subjects ($n = 10$ per group matched for age and sex). RBCs slides were prepared as previously described [10]. Briefly, after blocking of nonspecific reactive sites, RBCs were incubated overnight at 4°C with a monoclonal anti-eNOS (2.5 μg/mL) (BD Biosciences, Milano, Italy) or polyclonal anti-arginase I or monoclonal anti-arginase II (4 μg/mL, for both) (Santa Cruz Biotechnology, DBA Italia s.r.l., Milano, Italy) antibodies. After three washings, an anti-mouse or anti-rabbit AlexaFluor488 conjugated secondary antibody (Invitrogen, Life Technologies Italia, Monza, Italy) was added and the immune complexes were visualized by laser scanning confocal microscope (LSM710, Carl Zeiss, Milano, Italy) using a 63x/1.3 oil immersion objective lens. Images were captured and the fluorescence intensity (densitometric sum of grey) was quantified. Data are expressed as the mean level of fluorescence intensity, subtracted of negative control value obtained on the same slide in the absence of primary antibody. Multiple fields of view (at least three randomly selected areas) were captured for each slide.

2.6. Statistics and Scores Development. Numerical variables were summarized as mean and standard deviation (SD), unless otherwise stated, and categorical variables were summarized as frequencies and percentages. A sample size of 20 subjects per group allowed a statistical power of 90% to deem as significant a between-group difference in any analyte approximately equal to one standard deviation, with an alpha error of 0.05. Variables were compared between MVA and CAD or Ctrl by t-test or by covariance analysis, adjusting for age and sex. Variables with skewed distribution were log-transformed before analysis. Immunofluorescence intensity was compared between groups by repeated measures covariance analysis, taking into account replicate measures for each subject. All analyses were performed by SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA).

In order to provide a global indicator of all the variables related to NO pathway and to contain inflation of alpha error due to multiple testing, we developed a score similar to the OXY-SCORE, devised by our group few years ago [43]. First, to account for different measurement ranges and units, all the variables were standardized; that is, the mean was subtracted from individual values and the result was divided by the standard deviation. Second, the standardized values of the variables generally accepted as positively associated with endothelial function (Arg and Cit) were added, whereas standardized values of the variables negatively associated with endothelial function (ADMA, SDMA, MMA, and Orn) were subtracted. It is important to note that these associations were intended as "a priori" and were not inferred from the present study. We created a first score using variables measured in plasma (NO plasma score) and another score using variables measured in the RBCs (NO RBC score). Similarly, we created oxidative stress score, a simplified version of the OXY-SCORE including GSSG (with a plus sign) and GSH (with a minus sign).

3. Results

3.1. Population. The principal demographic and clinical characteristics of the two patient groups and of healthy subjects analyzed in this study are depicted in Table 1. No significant differences were found among groups except for age ($P = 0.01$ MVA versus CAD) that was considered as a confounder for group comparisons.

3.2. Biochemical Determinations of Metabolites Involved in Arg/NO Pathway and Oxidative Stress Status. In order to evaluate the potential impairment of Arg/NO pathway in MVA patients, we simultaneously measured the principal metabolites involved in this pathway, both in plasma and in the RBC compartment, and we compared them to the levels measured in CAD and in Ctrl (Table 2). In plasma, MVA patients showed Arg, Cit, and Orn levels similar to those of CAD patients and Ctrl. ADMA levels, instead, were higher in both MVA and CAD patients compared to Ctrl. SDMA and MMA levels did not differ among the three groups studied. In accordance to these findings, the Arg bioavailability (Arg/Orn + Cit ratio) was lower in MVA than in Ctrl and similar to CAD. In addition, the MVA Orn/Cit ratio, an index of activities of the Arg metabolic enzymes arginase and NOS, showed levels intermediate between those of CAD and Ctrl (Table 2).

In the RBC compartment, the levels of NO inhibitors ADMA and SDMA in MVA and CAD patients were higher than in Ctrl (Table 2). Interestingly, MMA levels were the highest in MVA. Arg bioavailability was similar in the three groups of subjects, whereas the Orn/Cit ratio was significantly lower in MVA than in CAD group but similar to Ctrl (Table 2).

Patients with MVA had higher levels of oxidative stress with respect to Ctrl, but lesser than those determined in CAD patients, as documented by the GSSG/GSH ratio measured in whole blood (Figure 1(a)). Specifically, both groups of patients showed lower levels of GSH and higher levels of GSSG with respect to Ctrl (Figure 1(b)).

Figure 2 shows the distribution of the analytes measured in plasma or RBCs of MVA and CAD patients expressed as fold change over Ctrl. In general, the analytes of the NO pathway behaved similarly in MVA and CAD and they were moderately elevated with respect to Ctrl, both in plasma and in RBCs. A special case is represented by MMA in RBCs, whose levels were higher in MVA with respect to Ctrl and CAD patients. As expected, the oxidative stress, in particular the oxidized form of glutathione, was higher in both MVA and CAD patients with respect to Ctrl.

3.3. Arginine Metabolic Enzymes: RBC-NOS and Arginase. The expression of RBC-NOS, visualized by immunofluorescence staining, revealed strong quantitative differences between both patient groups and Ctrl. RBCs of MVA and CAD patients had significantly lower RBC-NOS fluorescence, localized in the membrane and into the cytosol, with respect to Ctrl (Figure 3(a)).

The expression of both isoforms of arginase was also evaluated. RBCs of MVA patients and of Ctrl expressed lesser levels of arginase I than CAD patients ($P = 0.02$) (Figure 3(b)). In contrast, the expression of arginase II was not detectable in RBCs of Ctrl and of MVA or CAD patients (data not shown).

Table 1: Demographic and clinical characteristics of the subjects.

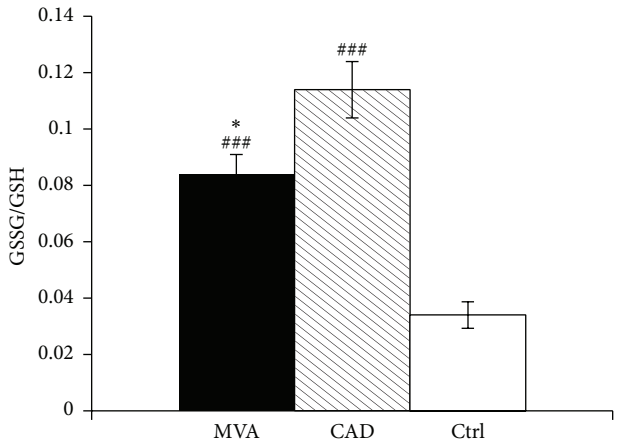
Variable	MVA (n = 25)	CAD (n = 22)	Ctrl (n = 20)
Age (years)	56.5 ± 10.3	66.1 ± 8.6	55.5 ± 10.2
Male gender	14 (56.0)	17 (77.3)	14 (70)
BMI	25.8 ± 3.3	27.3 ± 3.11	24.36 ± 2.35
Total cholesterol (mg/dL)	226.9 ± 59.2	208.3 ± 29.2	208.1 ± 26.8
HDL-cholesterol (mg/dL)	54.9 ± 17.3	46.9 ± 17.2	54.5 ± 17.3
LDL-cholesterol (mg/dL)	150.0 ± 52.4	133.8 ± 36.8	132.3 ± 22.1
Triglycerides (mg/dL)	105.5 ± 66.9	131.5 ± 69.4	95.5 ± 32.5
Systolic blood pressure (mmHg)	130.0 ± 13.2	138.9 ± 18.3	130.0 ± 14.0
Diastolic blood pressure (mmHg)	78.0 ± 8.3	80.0 ± 9.1	78.0 ± 6.0
Creatinine (mg/dL)	0.85 ± 0.22	0.86 ± 0.22	0.81 ± 0.14
Current smoker	3 (12.0)	3 (13.64)	0 (0)
Hypercholesterolemia	14 (56.0)	14 (63.6)	2 (12.5)
Hypertriglyceridemia	1 (4.0)	2 (9.1)	1 (6.25)
Hypertension	11 (44.0)	14 (63.6)	2 (12.5)
Pharmacological treatments			
Converting enzyme inhibitors	2 (8.0)	6 (27.3)	0 (0)
Antithrombotics	23 (85.1)	17 (77.3)	0 (0)
Beta-blockers	10 (40.0)	5 (23.8)	1 (5.88)
Calcium channel blockers	1 (4.0)	4 (18.2)	1 (5.88)
Diuretics	2 (8.0)	2 (9.1)	0 (0)
Statins	4 (16.0)	4 (18.2)	2 (11.8)
Hypoglycemics	0 (0)	0 (0)	0 (0)
Angiotensin receptor blockers	4 (16.0)	6 (27.3)	0 (0)

Quantitative variables are expressed as mean ± SD and categorical variables as n (%).

Table 2: Biochemical determinations in plasma and RBCs.

	Plasma			RBC		
	MVA (n = 25)	CAD (n = 22)	Ctrl (n = 20)	MVA (n = 25)	CAD (n = 22)	Ctrl (n = 20)
Arg	74.22 [69.15–87.08]	82.88 [64.81–95.74]	84.80 [73.13–98.01]	7.57 [5.00–9.53]	8.64 [5.31–10.48]	6.84 [3.86–7.80]
Cit	27.45 [24.21–31.16]	27.61 [20.41–33.83]	26.55 [24.13–30.68]	11.36 [9.58–12.35]	10.48 [8.36–12.11]	8.37 [7.04–10.78]
Orn	47.66 [41.19–54.11]	$51.82^{\#}$ [48.72–61.68]	40.84 [34.09–46.08]	39.48 [29.52–49.06]	42.59 [36.74–44.90]	33.93 [23.77–43.07]
ADMA	$0.51^{\#}$ [0.43–0.60]	$0.49^{\#}$ [0.45–0.59]	0.41 [0.35–0.47]	$0.18^{\#}$ [0.14–0.27]	$0.20^{\#}$ [0.15–0.25]	0.15 [0.12–0.20]
SDMA	0.47 [0.42–0.60]	0.54 [0.45–0.63]	0.42 [0.36–0.48]	$0.13^{\#\#}$ [0.09–0.16]	$0.13^{\#}$ [0.12–0.15]	0.10 [0.06–0.11]
MMA	0.12 [0.09–0.13]	0.11 [0.08–0.13]	0.12 [0.10–0.14]	0.13^{*} [0.07–0.18]	0.05 [0.04–0.09]	0.09 [0.05–0.12]
Arg bioavailability	$1.06^{\#}$ [0.90–1.26]	$1.01^{\#}$ [0.87–1.22]	1.25 [1.04–1.41]	0.13 [0.11–0.19]	0.16 [0.12–0.19]	0.14 [0.09–0.20]
Orn/Cit ratio	1.74 [1.42–2.21]	$1.97^{\#\#\#}$ [1.69–2.40]	1.48 [1.36–1.71]	3.88^{*} [3.10–4.41]	3.96 [3.51–5.03]	3.92 [3.20–4.32]

Quantitative variables are expressed as median [interquartile interval]. $^{*}P < 0.05$ versus CAD; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus Ctrl adjusted for age and gender after log-transformation of the data.



(a)

	MVA (n = 25)	CAD (n = 22)	Ctrl (n = 20)
GSH (μmol/g Hb)	6.40$^{\#}$ [4.90–7.20]	6.10$^{\#}$ [5.30–6.70]	7.60 [6.80–8.60]
GSSG (μmol/g Hb)	0.50$^{\#}$ [0.35–0.59]	0.62$^{\#}$ [0.50–0.72]	0.26 [0.21–0.32]

(b)

Figure 1: GSSG/GSH ratio in whole blood. The results are expressed as mean ± SE for GSSG/GSH ratio (a) or as median (interquartile range) (b) in whole blood from patients with microvascular angina (MVA n = 25) or coronary artery disease (CAD n = 22) or healthy subjects (Ctrl n = 20). $^{*}P < 0.05$ versus CAD, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus Ctrl.

3.4. Summary Scores of NO Pathway and Oxidative Stress. The analytes, that is, substrate, inhibitors, and enzymatic products involved in NO synthesis, were combined into appropriate scores (see Section 2) in order to summarize the Arg/NO pathway in the examined clinical settings. In Figure 4, the Cartesian plane was defined by the NO plasma score (x-axis) and the NO RBC score (y-axis); the intersection of the axes identifies the midpoint of the entire sample, and the units are expressed in terms of standard deviations. The Ctrl group was placed in the first quadrant (positive values for both scores), whereas the two patient groups were placed in the third quadrant (negative values for both scores). To be noticed, the MVA group was located in a more negative position, along the NO RBC score axis, compared with the CAD group; however, the difference did not reach statistical significance.

Figure 5 shows the Cartesian plane defined by the oxidative stress score (x-axis) and the NO plasma score (y-axis). In this graph, the control group was placed in the quadrant characterized by a negative oxidative score and by a positive NO score. In contrast, both groups of patients were placed in the quadrant relative to a positive oxidative score and a negative NO score, with the CAD group located in a more extreme position with respect to MVA (although this difference did not reach statistical significance: $P = 0.08$ for multivariate ANOVA).

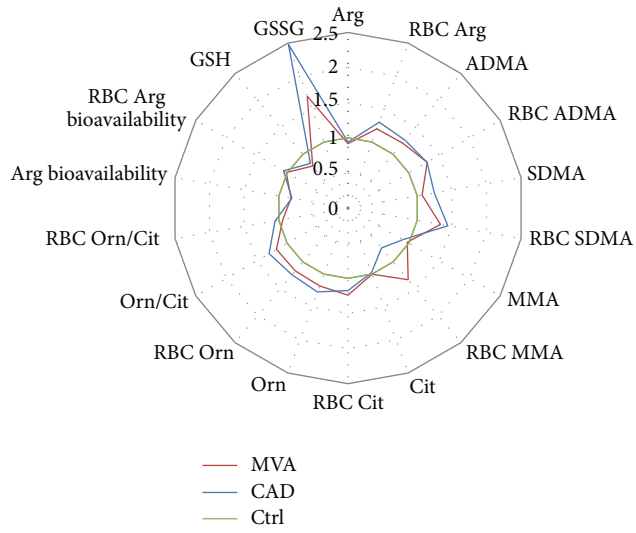


Figure 2: Levels of analytes involved in Arg/NO pathway measured in plasma and in RBCs isolated from patients with microvascular angina (MVA n = 25) or coronary artery disease (CAD n = 22) or healthy subjects (Ctrl n = 20). The results are expressed as fold change over Ctrl.

4. Discussion

The study described above shows for the first time that RBCs of patients with MVA contain higher levels of inhibitors of the NO synthesis than Ctrl and that these levels do not markedly differ from those found in CAD patients. A similar picture is found in plasma, as previously described by others [37, 38]. Finally, NOS expression in RBCs was found markedly reduced in both MVA and CAD patients. In addition, oxidative stress was found increased in both patient groups, mostly in CAD.

The pathophysiology of MVA is not completely understood yet, even if the several metabolic, haemodynamic, and vasospastic alterations have been linked to this syndrome. Recently, it has been reported that RDW values are significantly higher in both MVA and CAD patients compared to healthy subjects [36]. However, as documented by the absence of modifications in RDW values (data not shown), in our study, the impairment of NO pathway in RBCs of MVA patients is not associated with changes in the size of circulating RBCs. The RBCs of MVA patients, however, showed higher levels of NO synthesis inhibitors and this finding parallels the data found in plasma. As a consequence, in a Cartesian plane, defined by NO scores, the MVA group was located in a negative position along the NO RBC score axis with respect to Ctrl, thus suggesting a possible alteration in NO production, more pronounced in MVA with respect to CAD.

The limitation of our study might be the calculation of the NO scores without measuring NO itself. This highly reactive molecule and its active metabolites are influenced by several factors, including dietary nitrate intake and renal function, particularly in the plasma compartment. Thus, we

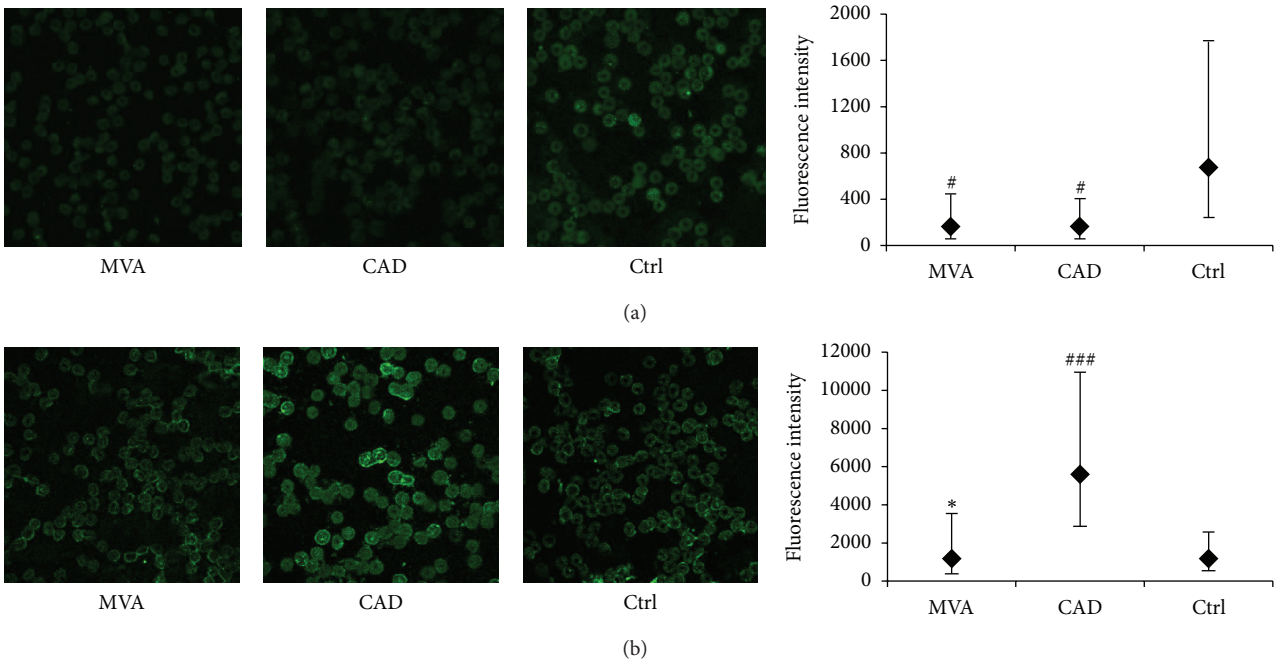


Figure 3: NO synthase and arginase expression in human RBCs. Representative immunofluorescent images (630x magnification) of RBCs isolated from patients with microvascular angina (MVA) or coronary artery disease (CAD) or healthy subjects (Ctrl), stained for RBC-NOS (a) or arginase I (b). Data are expressed as the mean of fluorescent intensity ± SD subtracted of the negative control value (at least three fields were analyzed, $n = 10$ subjects for each group). $^{*}P < 0.05$ versus CAD; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus Ctrl.

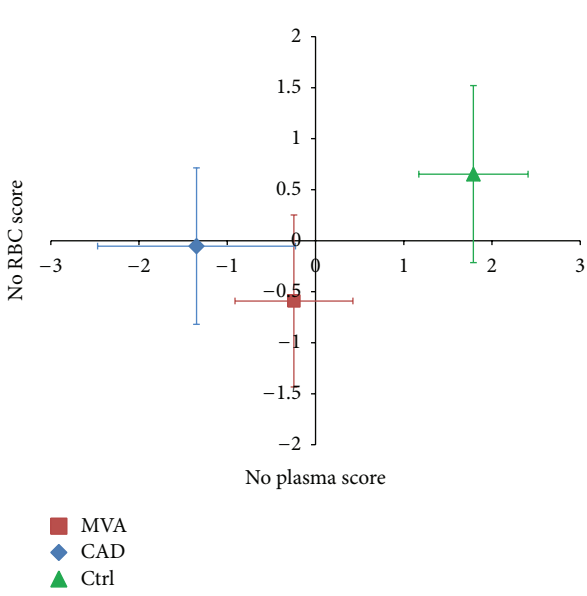


Figure 4: Arg/NO pathway score in plasma and in RBCs. The scores are computed after standardization of the analytes involved in NO synthesis (Arg, Cit, Orn, ADMA, SDMA, and MMA). The standardized values of the variables positively associated with endothelial function are added, whereas the values of variables negatively associated with endothelial function are subtracted. The NO score is calculated in plasma and in RBC compartment. NO RBC score: $P < 0.05$ MVA versus Ctrl; NO plasma score: $P < 0.001$ MVA versus Ctrl and CAD versus Ctrl.

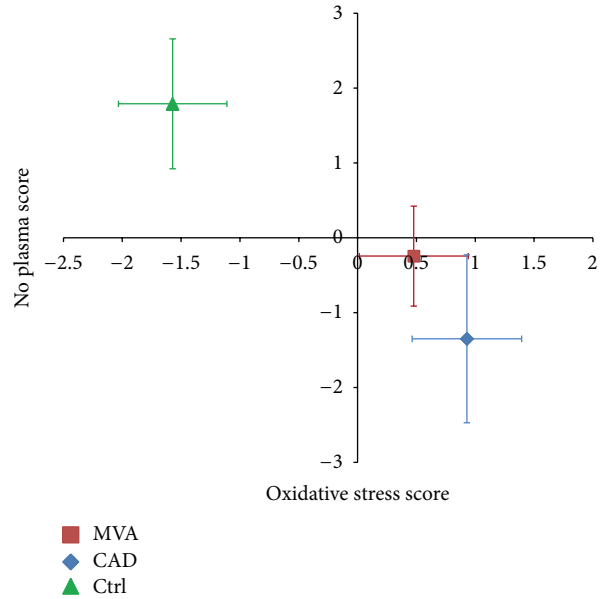


Figure 5: NO plasma and oxidative stress scores. The NO plasma score is computed after standardization of the analytes involved in NO synthesis (Arg, Cit, Orn, ADMA, SDMA, and MMA). The standardized values of the variables positively associated with endothelial function are added, whereas the values of variables negatively associated with endothelial function are subtracted. The oxidative stress score is calculated by the values of GSSG (plus sign) and GSH (minus sign). NO plasma score: $P < 0.001$ MVA versus Ctrl and CAD versus Ctrl; oxidative stress score: $P < 0.001$ MVA versus Ctrl and CAD versus Ctrl.

cannot exclude that other NOS independent factors may add additional information for an overall picture of this metabolic pathway in MVA.

Of interest is the observation that, similar to what previously described for CAD patients [10], we found a marked reduction in NOS expression in RBCs of MVA patients. This finding is of particular relevance because RBCs have a systemic impact in terms of NO production and may represent an important compartment, whose alteration participates to the reduction in the overall NO production.

Arg is the substrate for the NOS enzymes, including RBC-NOS, and it has been shown that an increase of substrate availability in the stenotic lesion induced dilation of the coronary artery segment [44]. Arg is also substrate for the arginase enzyme, whose activity is increased in different pathological conditions associated with a reduction of NO [17, 45].

Two different isoforms of arginase are identified in human and arginase I, which is the only arginase so far described in RBCs, accounts for about 98% of total blood arginase activity [46]. In our condition, greater amounts of arginase I in CAD patients, but not in MVA patients, were found. Since it has been reported that erythroid progenitor cells express both arginase I and arginase II [15], we measured also this enzyme in RBCs. According to the literature [17], we failed to detect measurable amounts of arginase II in Ctrl or in patients.

Increased erythrocyte arginase activity associated with lowered NO plasma levels and with impairment in erythrocytes has been reported in sickle cell disease patients [40]. Interestingly, the consumption of cocoa flavanols reduced the erythrocyte arginase activity, suggesting a possible therapeutic intervention by the regulation of Arg and NO bioavailability [47].

An important condition able to affect NO bioavailability is oxidative stress. Of relevance is the observation that the ratio between oxidized and reduced glutathione was almost doubled in whole blood of MVA patients, suggesting an increased oxidative stress in this condition. A role of oxidative stress in lowering NO bioavailability has been previously highlighted, but the information in MVA is still scanty [27, 48, 49]. We found a marked increase of GSSG/GSH, based on the increase of oxidized glutathione, which was even more pronounced in CAD patients and on a decrease of GSH in MVA patients. This observation is in accordance with data reported by Dhawan et al. [50], who showed a positive correlation between GSH levels and coronary flow velocity reserve, thus predicting impaired microvascular function.

Finally, the concomitant assessment of oxidative stress and NO pathway in patients indicates that both MVA and CAD patients are placed in the Cartesian plane quadrant relative to a positive oxidative score and a negative NO score, with the CAD group located in a more extreme position with respect to MVA.

Thus, as previously suggested by Rassaf and collaborators [51], a multiple-level approach by assessing biochemical, structural, and functional changes in the vasculature may be important for an early diagnosis of cardiovascular diseases and for a better characterization of this multifactorial disease.

5. Conclusion

Our study shows that changes in the Arg/NO metabolic profile, coupled to increases in oxidative stress, occur in MVA with a trend toward an impairment similar to that of CAD patients. In particular we have described for the first time alterations in the capacity of RBCs to produce NO in a pathological condition characterized mostly by alterations at the microvascular bed with no significant coronary stenosis.

Conflict of Interests

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

Authors' Contribution

Benedetta Porro and Sonia Eligini contributed equally to this paper as first authors.

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Research Article

New Insights into the Steen Solution Properties: Breakthrough in Antioxidant Effects via NOX2 Downregulation

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Ex vivo lung perfusion (EVLP) allows perfusion and reconditioning of retrieved lungs for organ transplantation. The Steen solution is specifically designed for this procedure but the mechanism through which it elicits its activity is still to be fully clarified. We speculated that Steen solution may encompass antioxidant properties allowing a reestablishment of pulmonary tissue homeostasis. Blood samples from 10 healthy volunteers were recruited. Platelets and white cells were incubated with Steen solution or buffer solution as control and stimulated with suitable agonists. Reactive oxidant species (ROS), soluble NOX2 (sNOX2-derived peptide), a marker of NADPH oxidase activation, $p47^{phox}$ translocation to cell membrane and isoprostanes production, as marker of oxidative stress, and nitric oxide (NO), a powerful vasodilator and antioxidant molecule, were measured upon cell stimulation. The Steen solution significantly inhibited $p47^{phox}$ translocation and NOX2 activation in platelets and white cells. Consistent with this finding was the reduction of oxidative stress as documented by a significantly lowered formation of ROS and isoprostanes by both platelets and white cells. Finally, cell incubation with Steen solution resulted in enhanced generation of NO. Herewith, we provide the first evidence that Steen solution possesses antioxidant properties via downregulation of NADPH oxidase activity and enhanced production of NO.

1. Introduction

Organ transplantation improves quality and expectancy of life of patients with end-stage organ failure. The claim for transplantation is expected to increase because of the prolonged expectancy of life. However, despite an exponential growth in the 90s, the absolute number of donors and their rate per million population (pmp) did not further improve [1, 2]. To overcome these hurdles and increase the pool of organs, national health systems along with health professionals directly involved in this field contributed to producing policies aimed at optimizing all phases of the process of organ donation and transplantation after brain death. However, this strategy, despite its proved efficacy, has partially failed to balance the organ offer to its growing demand [3].

In this context, lung transplantation (LT) is considered a viable therapeutic option for a selected group of patients with end-stage lung disease. However, the increasing number of patients on the waiting list clearly exceeds the number of available donors; this is because approximately only an average of 20% of the potential multiorgan donors is currently used for LT [4]. This contributes to an increase in waiting

time and mortality on the waiting list that often exceeds 20% [5]. Most of the reasons why the wide majority of the donor lungs are considered unsuitable for transplantation are related to lung injury occurring during trauma or after brain death and to the complications associated with a prolonged intensive care unit (ICU) stay. Consequently, a number of strategies have been proposed: the use of marginal donors [6], living related LT [7, 8], or non-heart beating donation [9]. Notwithstanding these improvements, the number of transplants has hitherto not significantly increased. Behind these assertions, the concept of lung procurement from marginal and extended donors and the possibility of "organ reconditioning" have been successfully used for transplantation and this new concept is gaining large acceptance.

Normothermic ex vivo lung perfusion (EVLP) allows perfusion of the retrieved lungs in an ex vivo circuit with functional reassessment and reconditioning before transplantation, irrespective of the protocol adopted: the Lund model or the Toronto model [9–11]. This novel technique utilizes an original priming/perfusion solution specifically designed for this procedure such as the Steen solution. In the Lund model, the EVLP procedure is performed with a mixture of Steen solution and washed erythrocytes in order to reach a hematocrit of 15%; besides, the left atrium is kept open, offering the possibility of a normal cardiac output (5-6 L/min) during EVLP. In the Toronto protocol, the EVLP procedure is carried out with an "acellular" Steen solution and a closed left atrium achieving thus only 40% of the normal cardiac output. Irrespective of the protocol adopted, the reason why this technique seems to work so profitably is mainly correlated to different variables: the use of steroids and high dose of antibiotics, the high osmotic power of the solution, and the effects of the leukocyte depletion filter interposed on the circuit. The EVLP procedure is a typical model of ischemia reperfusion which is associated with reactive oxidant species (ROS) formation; such a phenomenon may theoretically preclude optimal lung perfusion and consequently the most favorable clinical outcome as ROS has negative effect on endothelial function via inhibition of nitric oxide (NO) activity. We speculated that Steen solution may possess antioxidant properties which may blunt ROS formation thus improving lung perfusion. To address this issue, we explored an ex vivo experimental model consisting in challenging the Steen solution with blood cells activated to produce ROS generated by NOX2, the catalytic subunit of NADPH oxidase.

2. Material and Methods

2.1. Subjects. We studied ten healthy volunteers (5 males, 5 females, age: 33.5 ± 5.7) (Table 1). Written informed consent was obtained from all subjects. The study was approved by the local ethical committee (December 12, 2013, Protocol number 3010) and was conducted in accordance with the principles embodied in the Declaration of Helsinki.

Blood samples were obtained after a 12-hour fast between 8.00 and 9.00 a.m. from subjects undergoing routine biochemical analysis including total cholesterol and glucose. Samples, obtained from healthy subjects after supine rest for at least 10 min, were taken into tubes with 104 3.8% sodium citrate or EDTA.

Table 1: Characteristics of healthy subjects.

Patients	$N = 10$
Age (years)	32.8 ± 3.1
Males (%)	5 (50)
Body mass index (kg/m^2)	21.1 ± 4.5
Systolic blood pressure (mmHg)	126 ± 11
Diastolic blood pressure (mmHg)	79 ± 10
Total cholesterol (mg/dL)	184 ± 7
Low density lipoprotein cholesterol (mg/dL)	97 ± 10
Fasting glycemia (mg/dL)	85 ± 10
Smokers	No
Gas exchange assessed by spirometry test	Within normal ranges

2.2. Laboratory Analyses. All materials were from Sigma-Aldrich (Milan, Italy) unless otherwise specified. The Steen solution was purchased from Vitrolife (Göteborg, Sweden). It is a physiological salt solution containing human serum albumin (providing normal oncotic pressure in order to prevent edema formation), dextran (a mild scavenger which coats and protects endothelium from subsequent excessive leucocyte interaction and thrombogenesis), and characterized by a prevalent extracellular electrolyte composition (low potassium, decreasing free radical generation and preventing vascular spasm under normothermic conditions).

2.3. Platelet Preparation and Activation. To acquire platelet-rich plasma (PRP), samples were centrifuged for 15 minutes at 180 g. In order to avoid leukocyte contamination, only the top 75% of the PRP was collected according to Pignatelli et al. [12]. Platelet pellets (PLT) were obtained by double centrifugation (5 minutes, 300 g) of PRP. Acid/citrate/dextrose (ACD) (1 : 7 v/v) was added to avoid platelet activation during processing. Samples were suspended in HEPES buffer (buffer solution, BS) in presence of 0.1% albumin, pH 7.354 (2×10^5 PLT/mL, unless otherwise noted), or in presence of 1 mL of Steen solution (2×10^5 PLT/mL, unless otherwise noted) and stimulated with or without 0.5 mM arachidonic acid (AA) in presence or absence of the inhibitor of NADPH oxidase (apocynin, 50 μM) at room temperature for 15 minutes. Cells were separated from the supernatant by centrifugation (5 minutes, 300 g) and the two fractions, cells and supernatants, were stored at −80°C until analysis.

2.4. Human Polymorphonuclear Leukocyte Preparation and Activation. Polymorphonuclear leukocytes (PMNs) were isolated from freshly taken EDTA blood from healthy volunteers by dextran enhanced sedimentation of red blood cells, Ficoll-Histopaque density centrifugation, lysis of remaining erythrocytes with distilled water, and washing of cells with Hank's balanced salt solution (HBSS) (buffer solution, BS) in absence of any divalent cations. Finally, the cell pellet was suspended in 1 mL of HBSS or 1 mL of Steen solution at the final concentration of 1×10^6 cells/mL and stimulated with phorbol 12-myristate 13-acetate (PMA) (10 μM) in presence

or absence of the inhibitor of NADPH oxidase (apocynin, 50 μM) at room temperature for 15 minutes. Cells were separated from the supernatant by centrifugation (5 minutes, 300 g) and stored at −80°C until analysis.

2.5. Lymphocytes/Monocytes Preparation. Blood samples were collected in heparinized tubes (10 IU/mL). Lymphocytes/monocytes (LYM/MON) (1×10^6 cells/mL) were isolated after centrifugation of the blood from healthy volunteers ($n = 5$, healthy subjects) with a polysucrose-sodium diatrizoate solution, 1.077 g/mL density, and 280 mOsm osmolarity (Lymphoprep; Nycomed, Oslo, Norway) at 800 g at 20°C. The LYM/MON cell layer was collected and the cells were thus washed two times in a solution of cold phosphate-buffered saline (pH 7.2) (PBS), supplemented with 1% fetal calf serum and 2 mmol/L EDTA (Sigma-Aldrich). Finally, the cell pellet was suspended in 1 mL of PBS (buffer solution, BS) or 1 mL of Steen solution at the final concentration of 1×10^6 cells/mL. The cell suspension was stimulated with or without lipopolysaccharide (50 pg/mL) (LPS) in presence or absence of the inhibitor of NADPH oxidase (apocynin, 50 μM) at room temperature for 15 minutes. Cells were separated from the supernatant by centrifugation (5 minutes, 300 g) and stored at −80°C until analysis.

2.6. Assessment of Reactive Oxygen Species (ROS) Production by Flow Cytometry. Cells *suspensions* were incubated with 2′,7′-dichlorofluorescin diacetate (5 μM) (15 minutes at 37°C). 10 μL of each activated and unactivated sample was diluted with 1 mL of buffer solutions or Steen solution and analyzed by flow cytometry. ROS production was expressed as mean fluorescence (MF).

2.7. Analysis of sNOX2-dp and 8-iso-PGF2a-III. Extracellular levels of soluble NOX2-derived peptide (sNOX2-dp), a marker of NADPH oxidase activation, were detected by ELISA method as previously described by Pignatelli et al. [12]. The peptide was recognized by the specific monoclonal antibody against the amino acidic sequence (224–268) of the extra membrane portion of NOX2 (catalytic core of NADPH oxidase), which was released in the medium upon platelet activation. Values were expressed as pg/mL; intra-assay and interassay coefficients of variation were 5.2% and 6%, respectively. Cells 8-iso-PGF2α-III production was measured by EIA assay method (Tema Ricerca, Italy) and expressed as pmol/L. Intra- and interassay coefficients of variation were 5.8% and 5.0%, respectively.

2.8. Cells NOx Measurement. A colorimetric assay kit (Tema Ricerca, Italy) was used to determine the nitric oxide metabolites nitrite and nitrate (NOx) in the supernatant of platelet and white cells in presence or absence of the Steen solution (1 mL). Intra-assay and interassay coefficients of variation were 2.9% and 1.7%, respectively.

2.9. Membrane and Cytoplasmic Proteins Extraction. To analyze the specificity of Steen solution in blocking cells NADPH oxidase activation, the effect of this solution was analyzed on the translocation of $p47^{phox}$ from cytosol to membranes in agonists-activated cells according to Fortuño et al. [13].

Briefly, the extraction of membrane and cytoplasmic proteins was performed by using the ProteoJET Membrane Protein Extraction Kit (Fermentas International Inc., Maryland, USA) [13].

2.10. Western Blot Analysis of $p47^{phox}$. Activated and unactivated samples were suspended in a 2X lysis buffer (5 mM EDTA, 0.15 mol NaCl, 0.1 mol Tris, pH 8.0, and 1% triton and protease inhibitor cocktail). Equal amounts of protein (30 μg/lane) estimated by Bradford assay were solubilized in a 2X Laemmli buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-$p47^{phox}$ (1 μg/mL) incubated overnight at 4°C. Immune complexes were detected by enhanced chemiluminescence. The developed spots were calculated by densitometric analysis on a NIH image 1.62f analyzer and the value was expressed in arbitrary unit (AU).

2.11. Statistical Analysis. Continuous variables are reported as mean ± standard deviation and categorical variables as n (%). Continuous variables were compared with Student's t-test and ANOVA, as appropriate. Statistical significance was set at the 0.05 2-tailed level, with P values unadjusted for multiplicity unreported throughout. Computations were performed with SPSS 20 (IBM, Armonk, NY, USA).

2.12. Sample Size. We computed the minimum sample size with respect to a two-tailed one-sample paired Student's t-test, considering the following: (i) that $|\delta| \geq 8$ pg/mL would be a clinically relevant difference in NOX2 levels between stimulated cells treated without or with Steen solution, (ii) a 5 pg/mL standard deviation (SD) for the paired difference; (iii) type-I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$. These assumptions lead to $n = 9$.

3. Results

3.1. Role of the Steen Solution in ROS Production and 8-iso-PGF2a-III Production. The production of ROS in PLT, PMNs, and LYM/MON was evaluated by flow cytometric analysis using the fluorescent probe 2′,7′-dicloroflosceinaacetate (DCFH-DA). As a positive control of ROS inhibition, we repeated the experiments with apocynin, the specific inhibitor of NADPH oxidase.

As shown in Figures 1(a), 1(b), 1(c), and 1(d), we observed an increased ROS production in cells suspended in the buffer solution and stimulated with AA (0.5 mM) for PLT, LPS (50 pg/mL) for LYM/MON, and PMA (10 μM) for PMNs compared to nonstimulated cells. In presence of Steen solution (1 mL), we observed a reduction of ROS production in PLT (22.6 ± 4.1 MF versus 36.2 ± 8.0 MF; $P < 0.0001$), LYM/MON (28.6 ± 4.5 MF versus 46.6 ± 6.0 MF; $P < 0.0001$), and PMNs (31.0 ± 4.7 MF versus 50.8 ± 7.1 MF; $P < 0.0001$) compared to stimulated cells in buffer solutions. The positive control, apocynin, significantly inhibited the production of ROS (Figures 1(a), 1(b), and 1(c)) ($P < 0.0001$).

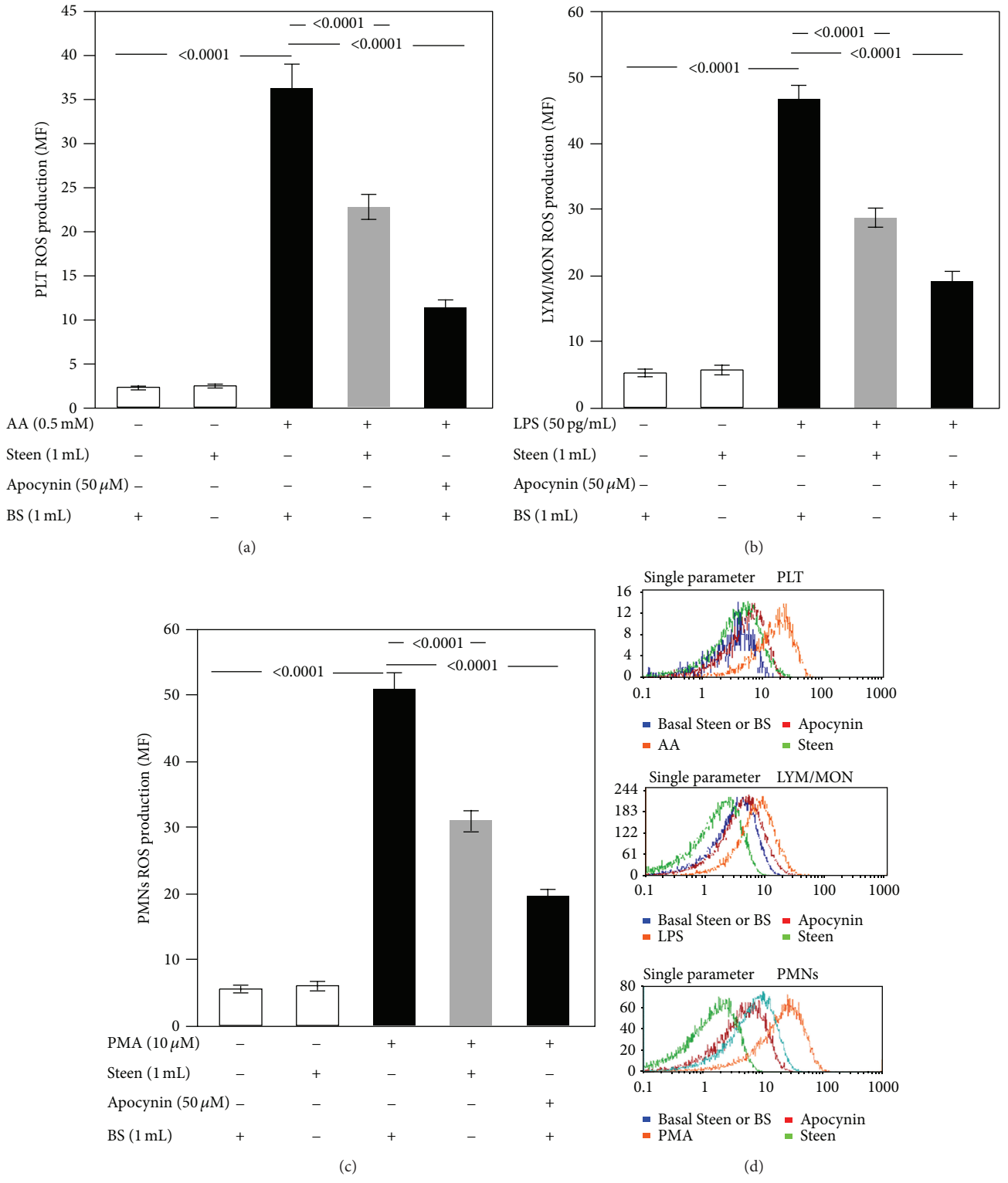

Figure 1: Role of Steen solution in the cellular production of ROS. In vitro study: ROS production in cells suspended in the buffer solution (BS) or Steen solution and stimulated with or without arachidonic acid (AA) (0.5 mM) for platelet (PLT) (a), lipopolysaccharide (LPS) (50 pg/mL) for lymphocytes/monocytes (LYM/MON) (b), and phorbol 12-myristate 13-acetate (PMA) (10 μM) for polymorphonuclear leukocytes (PMNs) (c). Cells were treated or not with apocynin (50 μM). Experiments were led on 10 subjects. White bars in the histogram graph represent control. A representative cytometry analysis of reactive oxygen species (ROS) production (d).

Coincidentally, with enhanced ROS formation, 8-iso-PGF2α-III production increased in cells suspended in buffer solutions and stimulated with AA (0.5 mM) for PLT, LPS (50 pg/mL) for LYM/MON, and PMA (10 μM) for PMNs compared to nonstimulated cells (Figures 2(a), 2(b), and 2(c)). Even in this case the Steen solution (1 mL) was able to reduce 8-iso-PGF2α-III production in PLT (112.3 ± 9.1 pmol/L versus 209.7 ± 10.3 pmol/ L; $P < 0.0001$), LYM/MON (136.6 ± 24.4 pmol/L versus 239.9 ± 24.1 pmol/L; $P < 0.0001$), and PMNs (192.2 ± 33.1 pmol/L versus 327.6 ± 25.1 pmol/L; $P < 0.0001$) (Figures 2(a), 2(b), and 2(c)) compared to stimulated cells in buffer solutions. The positive control, apocynin, significantly inhibited the production of 8-iso-PGF2α-III (Figures 2(a), 2(b), and 2(c)) ($P < 0.0001$).

3.2. Effect of the Steen Solution on NADPH Oxidase. To analyze the pathway involved in oxidative stress inhibition by the Steen solution, we studied the activation of NADPH oxidase by measuring the extra membrane portion of NOX2 (the catalytic core of NADPH oxidase), which is released in the medium upon cell activation, and $p47^{phox}$ expression on cell membrane, a key subunit for NADPH oxidase activation [14].

As shown in Figures 3(a), 3(b), and 3(c), Steen solution significantly decreased the activation of NADPH oxidase in each cell type (10.8 ± 2.4 pg/mL versus 20.1 ± 3.1 pg/mL in PLT, $P < 0.0001$; 17.6 ± 4.7 pg/mL versus 25.4 ± 4.3 pg/mL in LYM/MON, $P = 0.0002$; and 22.1 ± 4.7 pg/mL versus 31.1 ± 3.5 pg/mL in PMNs, $P < 0.0001$) as observed by reduced levels of sNOX2-dp, compared to stimulated cells in buffer solutions. The positive control, apocynin, significantly inhibited the activation of NADPH oxidase by reducing the levels of sNOX2-dp (Figures 3(a), 3(b), and 3(c)) ($P < 0.0001$).

Concerning the translocation of $p47^{phox}$ on cell membrane, we observed that Steen solution inhibited the translocation of this subunit of NADPH oxidase in all cell types (19.6±1.5 AU versus 28.3±3.5 AU in PLT, $P = 0.003$; 24.6±3.5 AU versus 34.3 ± 7.1 AU in LYM/MON, $P = 0.02$; and 26.6 ± 6.1 AU versus 41.6 ± 3.5 AU in PMNs, $P = 0.003$) compared to stimulated cells in buffer solutions (Figures 4(a), 4(b), 4(c), and 4(d)). The positive control, apocynin, significantly inhibited the expression of $p47^{phox}$ (Figures 4(a), 4(b), 4(c), and 4(d)) ($P < 0.0001$).

Cells suspended in buffer solution and incubated with the specific agonists significantly reduced NO generation compared with nonactivated cells (10.1 ± 2.5 versus 29.3 ± 6.4 μM in PLT, $P < 0.0001$, Figure 5(a); 10.6±2.7 versus 33.5± 4.9 μM in LYM/MON, $P < 0.0001$, Figure 5(b); and 14.4 ± 3.4 versus 34.8 ± 4.1 μM in PMNs, $P < 0.0001$, Figure 5(c)). Cell incubation with Steen solution enhanced NO generation (+44% for PLT, $P = 0.001$; +47% for LYM/MON, $P < 0.0001$; and +35% for PMNs, $P = 0.0002$) compared to stimulated cells in buffer solutions. Inhibition of oxidative stress by apocynin elicited NO overgeneration by activated cells (Figures 5(a), 5(b), and 5(c)).

4. Discussion

We report for the first time that the Steen solution reveals antioxidant properties by downregulating ROS-derived NOX2 activation.

ROS are chemically unstable molecules which rapidly react with other molecules giving rise to oxidized products such as oxidized LDL, peroxynitrite, or protein adducts [15]. At physiologic concentration, ROS serve as second messengers and, accordingly, they behave as intracellular signals for cell activation. Among the cell types in which ROS act as second messengers, platelets represent a typical example of ROS involvement in the process of activation. Thus, upon activation by common agonists, platelets produce several types of ROS such as superoxide anion or hydrogen peroxide that in turn contribute to propagation of platelet aggregation [16]. There are several enzymes that, upon activation, produce ROS, including myeloperoxidase, NADPH oxidase, xanthine-oxidase, or uncoupled eNOS. Among them, however, NADPH oxidase is the most important cell producer of ROS. Platelets possess all the subunits of the NADPH oxidase including gp91phox (NOX2) that is its catalytic subunit [17–19]. Activation of platelet NADPH oxidase is crucial for O_2^- production as shown by its complete suppression in case of NADPH oxidase hereditary deficiency. Regarding this, we have shown that platelets from patients with chronic granulomatous disease (X-CGD) have an almost complete suppression of platelet O_2^- production as a consequence of the hereditary deficiency of NOX2 [18]. Using blood cells as a tool to explore if the Steen solution has antioxidant properties, we found that two markers of oxidative stress, namely, ROS and isoprostane formation, were reduced upon cell incubation with the solution. Further support to the antioxidant property of the Steen solution was provided by demonstrating an increase of NO in the supernatant of stimulated cells incubated with the Steen solution. Thus, NO is a powerful vasodilator and antiaggregating molecule and is rapidly inactivated by ROS, which ultimately determine impaired NO generation.

Upon activation, blood cells release F2-isoprostanes, in particular 8-iso-PGF2α, a chemically stable compound derived from nonenzymatic oxidation of arachidonic acid (AA) [19]. We recently demonstrated that formation of platelet isoprostanes depends upon NADPH oxidase activation and contributes to the process of platelet recruitment via activation of the GpIIb/IIIa [17]. Thus, the reduction of isoprostanes yielded by the Steen solution leads us to hypothesize that such effect could be related to NADPH oxidase downregulation. Accordingly, we found that Steen solution inhibited NOX2 activation as shown by the significant lowering of sNOX2-dp in the supernatant of stimulated cells incubated with the Steen solution. This finding was corroborated by experiments through which we could demonstrate that the Steen solution impaired the translocation on platelet membrane of the cytosolic subunit $p47^{phox}$, which has a crucial role in the assembly process of NADPH subunits and is, therefore, essential for NOX2 activation. Taken together, these findings suggest that, in blood cells, the Steen solution could act as an antioxidant via downregulation of NOX2. Although we do not have the data, we can speculate that Steen solution will also act on erythrocytes. We cannot excclude that other enzymatic pathways may be implicated in the impaired ROS production elicited by the Steen solution. Furthermore, the exact mechanism through which the Steen

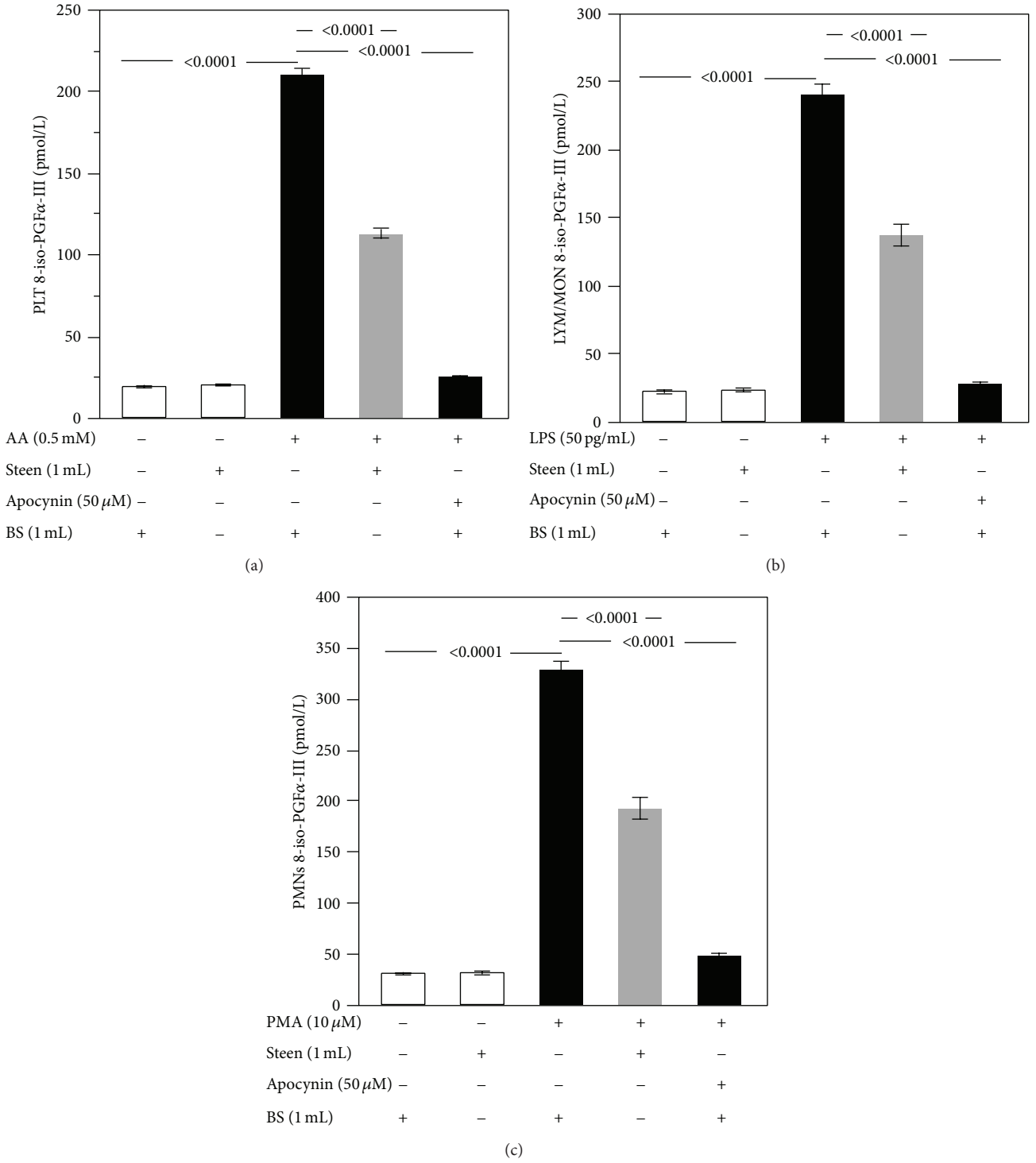

Figure 2: Role of Steen solution in the cellular production of 8-iso-PGF2α-III. In vitro study: 8-iso-PGF2α-III production in cells suspended in the buffer solution (BS) or Steen solution and stimulated with or without arachidonic acid (AA) (0.5 mM) for platelet (PLT) (a), lipopolysaccharide (LPS) (50 pg/mL) for lymphocytes/monocytes (LYM/MON) (b), and phorbol 12-myristate 13-acetate (PMA) (10 μM) for polymorphonuclear leukocytes (PMNs) (c). Cells were treated or not with apocynin (50 μM). Experiments were led on 10 subjects. White bars represent control.

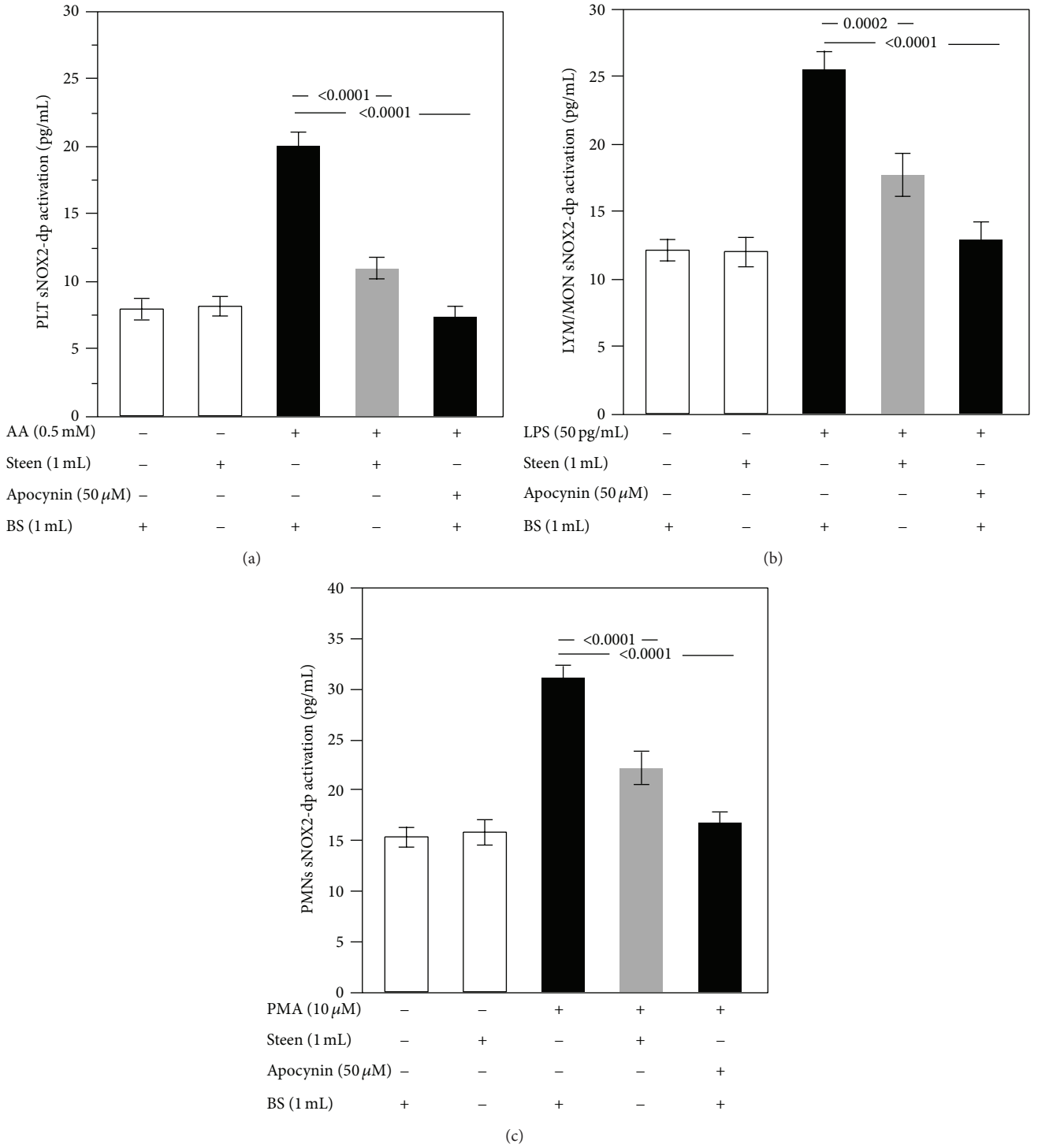

Figure 3: Role of Steen solution in the cellular NADPH oxidase activation. In vitro study: NADPH oxidase activation, evaluated by the release of sNOX2-dp, in cells suspended in the buffer solution (BS) or Steen solution and stimulated with or without arachidonic acid (AA) (0.5 mM) for platelet (PLT) (a), lipopolysaccharide (LPS) (50 pg/mL) for lymphocytes/monocytes (LYM/MON) (b), and phorbol 12-myristate 13-acetate (PMA) (10 μM) for polymorphonuclear leukocytes (PMNs) (c). Cells were treated or not with apocynin (50 μM). White bars represent control.

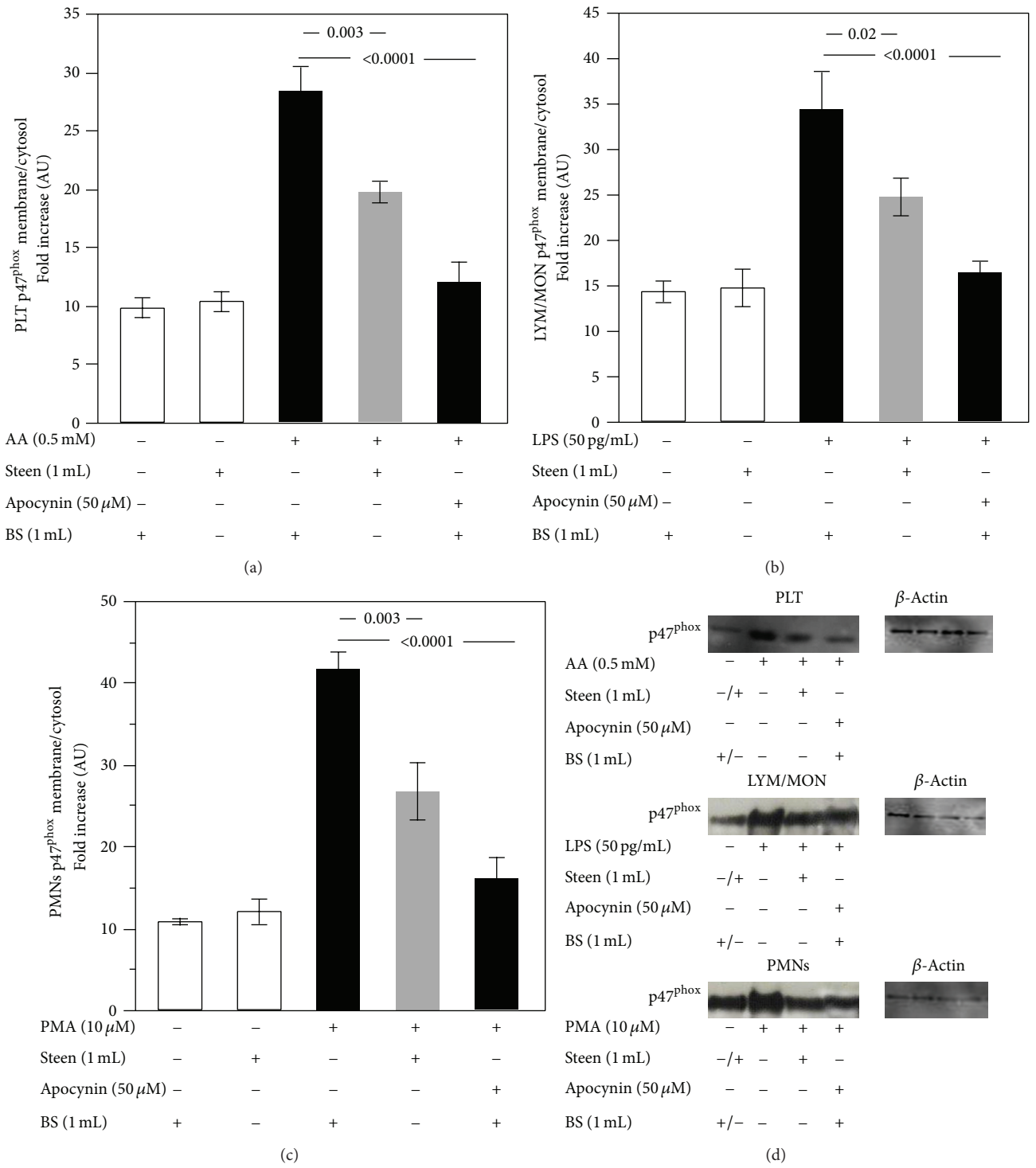

Figure 4: Role of Steen solution in the cellular $p47^{phox}$ translocation. In vitro study: $p47^{phox}$ translocation in cells suspended in the buffer solution (BS) or Steen solution and stimulated with or without arachidonic acid (AA) (0.5 mM) for platelet (PLT) (a), lipopolysaccharide (LPS) (50 pg/mL) for lymphocytes/monocytes (LYM/MON) (b), and phorbol 12-myristate 13-acetate (PMA) (10 μM) for polymorphonuclear leukocytes (PMNs) (c). Cells were treated or not with apocynin (50 μM). A representative Western blot analysis of membrane $p47^{phox}$ (d). White bars represent control.

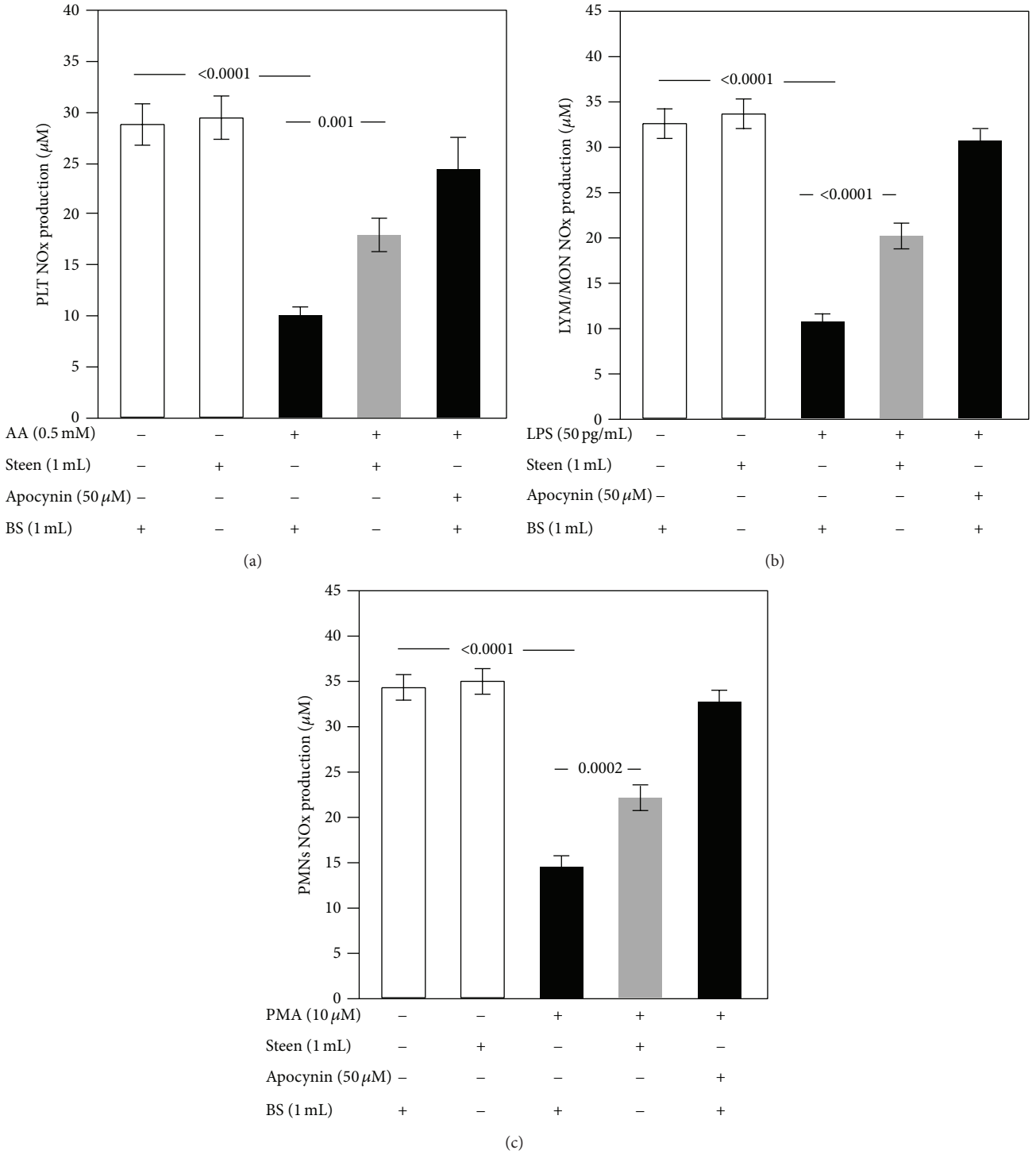

Figure 5: Role of Steen solution in the cell nitric oxide biodisponibility. In vitro study: nitric oxide biodisponibility, evaluated by the production of nitrite/nitrate (NOx), in cells suspended in the buffer solution (BS) or Steen solution and stimulated with or without arachidonic acid (AA) (0.5 mM) for platelet (PLT) (a), lipopolysaccharide (LPS) (50 pg/mL) for lymphocytes/monocytes (LYM/MON) (b), and phorbol 12-myristate 13-acetate (PMA) (10 μM) for polymorphonuclear leukocytes (PMNs) (c). Cells were treated or not with apocynin (50 μM). White bars represent control.

solution downregulates NOX2 is an open issue that deserves further investigation. Finally, it is arguable that the Steen solution could act as antioxidant also at endothelial level, but this hypothesis must be further explored.

In conclusion, our data suggests that the Steen solution plays an important antioxidant role by impairing the formation of ROS-derived NOX2 activation in several cell types. This finding provides new insights into the mechanism through which the Steen solution allows lung reconditioning during EVLP.

Further in vivo studies are currently ongoing and will shed additional lights on the translational value of the approach hereby proposed.

Conflict of Interests

The authors confirm that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Production of Nitric Oxide, IL-6, and TNF-Alpha in Palmitate-Stimulated PBMNCs Is Enhanced through Hyperglycemia in Diabetes

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We examined nitric oxide (NO), IL-6, and TNF-α secretion from cultured palmitate-stimulated PBMNCs or in the plasma from type 2 diabetes mellitus (T2MD) patients or nondiabetic (ND) controls. Free fatty acids (FFA) have been suggested to induce chronic low-grade inflammation, activate the innate immune system, and cause deleterious effects on vascular cells and other tissues through inflammatory processes. The levels of NO, IL-6, TNF-α, and MDA were higher in supernatant of palmitate stimulated blood cells (PBMNC) or from plasma from patients. The results obtained in the present study demonstrated that hyperglycemia in diabetes exacerbates *in vitro* inflammatory responses in PBMNCs stimulated with high levels of SFA (palmitate). These results suggest that hyperglycemia primes PBMNCs for NO, IL-6, and TNF-alpha secretion under *in vitro* FFA stimulation are associated with the secretion of inflammatory biomarkers in diabetes. A combined therapy targeting signaling pathways activated by hyperglycemia in conjunction with simultaneous control of hyperglycemia and hypertriglyceridemia would be suggested for controlling the progress of diabetic complications.

1. Introduction

Circulating free fatty acids (FFAs) are elevated in patients with type 2 diabetes mellitus (T2DM), obesity, metabolic syndrome, and dyslipidemia [1–4]. FFAs represent a complex group of structurally variable molecules stored in the body as triglycerides and released through lipolysis [3, 5]. FFAs are classified according to the carbon chain length in short-, medium-, and long-chain fatty acids, the presence or absence of double bonds as saturated (SFA) and unsaturated fatty acids, respectively, and the number of double bonds as mono- or polyunsaturated (PUFA) [6, 7]. The effect of FFA on cellular signaling pathways depends on the chemical structure. It has been reported that chronic exposure to SFA increases oxidative stress and inflammation, leading to the development of cardiovascular diseases and insulin resistance [8–12].

Oxidative stress, reflecting an imbalance between prooxidant and antioxidant effectors, plays an important role in diabetic vascular complications [13]. Superoxide, nitric oxide, and lipid peroxidation are indicators of oxidative stress in the body. Despite the number of studies concerning FFA-induced superoxide overproduction [14–22], there are few reports concerning FFA-induced nitric oxide (NO) production. NO is a highly diffusible and unstable gas that acts as a modulator of vascular tone, glucose transport in skeletal muscle cells and adipocytes, blood flow, force generation in skeletal muscle, cytotoxicity, and inflammation [23–26].

FFA also regulates the immune system through interactions with specific cell surface receptors, such as Toll-like receptors (TLR) and G-protein-coupled receptors (GPCR), thereby activating NF-kappaB and c-Jun amino-terminal kinase (JNK) pathways, which stimulate the secretion of proinflammatory cytokines (IL-1beta, IL-6 and TNF-alpha) and chemokines [27–30].

It is well known the effects of hyperglycemia and hyperlipidemia on peripheral blood mononuclear cells (PBMNCs) by activation of NADPH oxidase system leading to reactive oxygen species production, TLR expression, enhancing NF-kappaB activity, and inducing proinflammatory cytokines, chemokines, and circulating adhesion molecules secretion [8, 21, 31–41].

Thus, elevated plasma FFA levels act as inflammatory inducers, which potentially contribute to vascular disorders [27–30, 42, 43]. Thus, the aim of the present study was to investigate the *in vitro* effects of palmitate (C16:0), the major SFA in plasma [44, 45], on the modulation of oxidative stress and inflammation in T2DM patients. Nitric oxide, with or without palmitate induction, was quantified and correlated with proinflammatory cytokines secreted in the cultured supernatant of PBMNCs from type 2 diabetes patients. The association among plasmatic triglycerides, NO, proinflammatory cytokines (IL-6 and TNF-alpha), and oxidative stress (malondialdehyde) is discussed.

Table 1: Clinical and biochemical characteristics of the studied population.

Parameters	T2DM ($n = 29$)	ND ($n = 16$)	P
Female/Male ratio	19/10	11/5	NA
Age (years)	58.3 ± 9.0	57.1 ± 10.0	ns
Body mass index (kg/m^2)	30.8 ± 9.8	24.6 ± 4.1	<0.05
Disease duration (years)	6.7 ± 6.4	NA	NA
Systolic pressure (mmHg)	127.9 ± 14.5	122.3 ± 15.9	ns
Diastolic pressure (mmHg)	86.6 ± 8.6	88.9 ± 7.9	ns
Fasting glucose (mg/dL)	147.0 ± 40.7	89.0 ± 9.0	<0.05
Glycated hemoglobin (%)	8.1 ± 1.1	5.3 ± 0.2	<0.05
Total cholesterol (mg/dL)	191.6 ± 65.7	160.7 ± 20.0	ns
Low density lipoprotein (mg/dL)	115.3 ± 39.7	104.5 ± 32.6	ns
High density lipoprotein (mg/dL)	45.6 ± 10.6	50.2 ± 14.0	ns
Triglycerides (mg/dL)	142.0 ± 51.0	108.6 ± 37.7	<0.05

Data as means ± SD.
NA: not applicable; ns: not significant.
Significant differences between the groups were determined using Student's t-test ($P < 0.05$).

2. Material and Methods

This study was approved through the Ethical Committee of Santa Casa Hospital (Belo Horizonte-MG, Brazil) and written informed consent was obtained from all participants prior to the study.

2.1. Subjects. T2DM patients ($n = 29$), diagnosed according to the criteria of the American Diabetes Association [46], and nondiabetic controls ($n = 16$), ranging from 45 to 70 years of age, were recruited from the Endocrinology Department of Santa Casa Hospital. Type 2 DM patients were treated with statins and beta-blockers in addition to hypoglycemic drugs. Prior to the study, all volunteers received complete physical examinations, and detailed evaluations of medical histories and laboratory analyses were performed (Table 1). Pregnant women and individuals suffering from alcoholism, infection, inflammation, dementia, or malignant diseases and smoking addictions were excluded from this study.

2.2. Preparation of Fatty Acids. Palmitate and low-endotoxin bovine serum albumin (BSA, FFA-free) were purchased from Sigma-Aldrich Co. FFA was dissolved in 0.1 M NaOH at 70°C and subsequently complexed with 10% BSA at 55°C for 10 min to obtain a final FFA concentration of 500 μM (molar ratio 2.4 : 1) [42, 47]. A 10 mM fatty acid-albumin complex stock solution and a 0.5 μM BSA control solution were freshly prepared, filtrated, and diluted prior to each experiment.

2.3. Preparation of Peripheral Blood Mononuclear Cells. PBMNCs were purified from 10.0 mL of heparinized venous blood, using a Ficoll-Hypaque gradient as previously described [48], with slight modifications. The trypan blue exclusion test showed that the cell viability in all samples was >95%.

2.4. Preparation of Plasma. EDTA venous blood samples were collected using a standard venipuncture technique. The plasma was obtained through centrifugation (200 g for 15 min, at room temperature), and the samples were stored at −80°C until further analysis. Subsequent analyses were performed within 3 months from the day of storage.

2.5. Quantification of Proinflammatory Cytokines and NO in Supernatant of PBMNCs. Aliquots (100 μL) of a PBMNC suspension (1×10^6/mL) from T2DM patients and ND controls in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were incubated in the presence or absence of BSA (0.5 μM) or palmitate (500 μM) for 72 hours at 37°C under 5% CO_2. The final volume was adjusted to 300 μL in DMEM supplemented with 10% FBS. After incubation, the cells were centrifuged and the supernatant was collected. The interleukin-6 (IL-6 human EIA Kit—Enzo Life Sciences, Inc., New York, USA) and tumor necrosis factor-alpha (TNF-α human EIA Kit—Enzo Life Sciences, Inc., New York, USA) concentrations were determined through enzyme-linked immunosorbent assay (ELISA). Because NO is unstable, the quantitative of NO was indirectly determined based on the detection of the blood nitrite and nitrate levels. The NO concentration was measured using the Total Nitric Oxide Assay Kit (Assay Designs, Enzo Life Sciences, Inc., New York, USA).

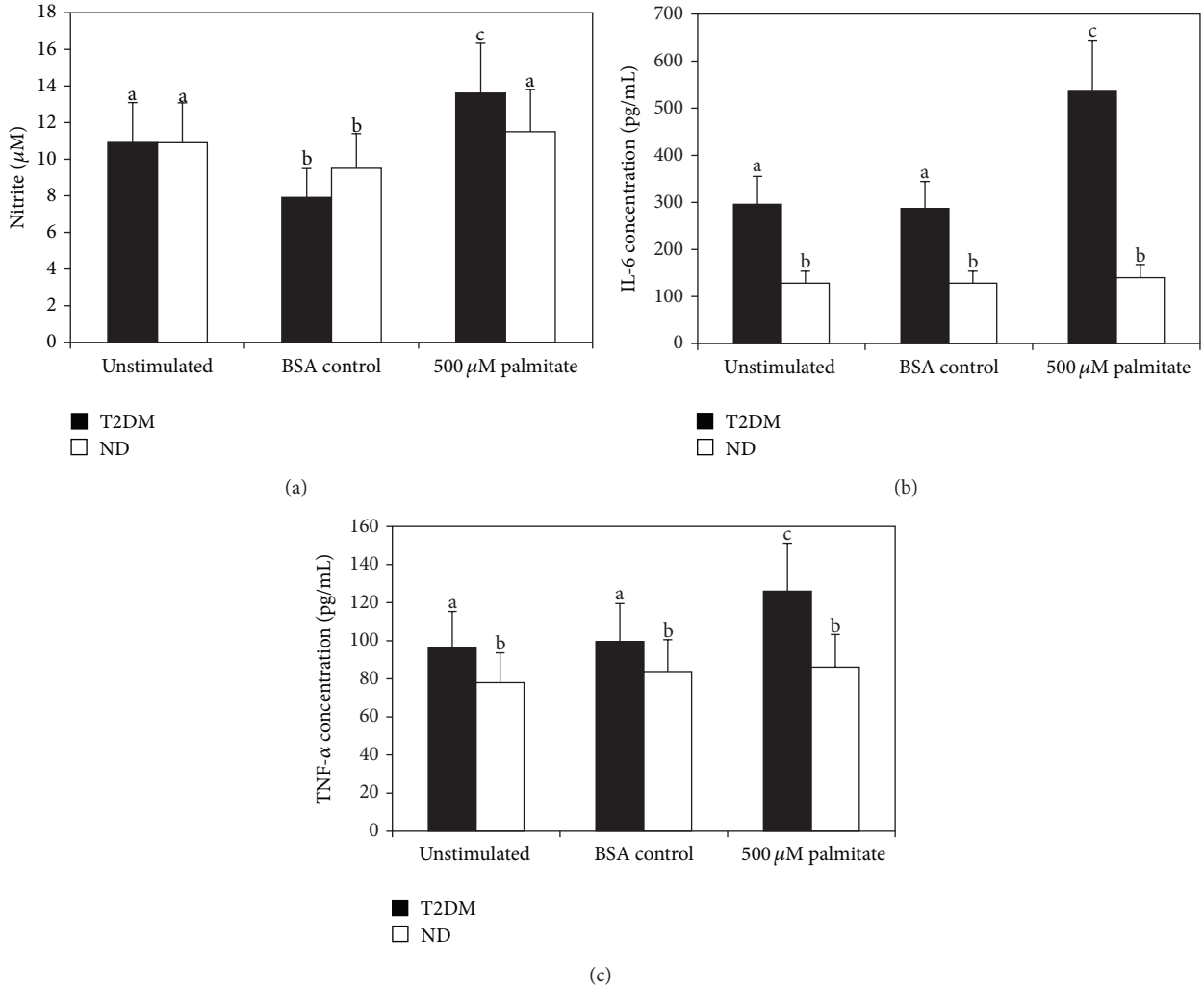

Figure 1: Palmitate induces NO, IL-6, and TNF-alpha secretion in peripheral blood mononuclear cells (PBMNC) from patients with type 2 diabetes. (a) Nitrite production; (b) IL-6 production; (c) TNF-alpha production. Different letters denote significance at $P < 0.05$ using Student's t-test. $n = 10$ for each group.

2.6. Quantification of NO, MDA, and Proinflammatory Cytokines in Plasma. The plasma levels of NO, IL-6, and TNF-alpha were determined as described above. The plasma MDA concentration was measured using the TBARS Assay Kit (ZeptoMetrix Corp., New York, USA) according to the manufacturer's instructions.

2.7. Statistical Analyses. The values are presented as the means ± standard deviation (SD). The nonparametric Kolmogorov-Smirnov test was used to assess the normal distribution of the continuous variables. Comparisons between groups were performed using unpaired Student's t-tests. Within-group correlations were performed using Pearson's r correlation. All analyses were considered significant at P values < 0.05 using Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA).

3. Result

3.1. PBMNCs from T2DM Patients Are More Sensitive to Palmitate Stimulation Than the Cells from ND Controls. As depicted in Figure 1, palmitate activated the secretion of NO, IL-6, and TNF-alpha in PBMNCs from T2DM patients compared with those from ND controls ($P < 0.05$). The results of the induced effect of palmitate on PBMNCs from T2DM patients and ND controls, expressed as the means ± SD, were NO, 11.5 ± 1.3 and 13.6 ± 2.2; IL-6, 86.1 ± 14.1 and 126.0 ± 29.0; and TNF-alpha, 140.0 ± 28.1 and 535.8 ± 115, respectively. The results shown in Figure 1 also demonstrated that PBMNCs from T2DM patients secreted significantly ($P < 0.05$) higher amounts of IL-6 (256.7 ± 81.1) and TNF-alpha (96.1 ± 17.5) compared with the cells from ND controls (IL-6: 128.3 ± 32.3, TNF-alpha: 78.0 ± 13.6). No difference ($P > 0.05$) was observed in NO production in PBMNCs

from T2DM patients (10.9 ± 1.7) and ND controls (10.9 ± 1.2) without stimulation.

The production of NO and proinflammatory cytokines was not altered in the presence of BSA ($P > 0.05$) in T2DM patients and ND controls: NO, 11.5 ± 1.3 and 13.6 ± 2.2; IL-6, 86.1 ± 14.1 and 126.0 ± 29.0; and TNF-alpha, 140.0 ± 28.1 and 535.8 ± 115, respectively.

3.2. Palmitate-Induced NO and IL-6 Production in PBMNCs Are Associated in T2DM Patients, but Not in ND Controls. Figure 2 shows the Pearson's correlations between the levels of NO, IL-6, and TNF-alpha in PBMNCs from T2DM patients and ND controls after palmitate stimulation. The correlation between NO and IL-6 were significantly strong in stimulated PBMNCs from T2DM patients ($r = 0.63$, $P = 0.04$) and moderate in PBMNCs from ND ($r = 0.47$, $P = 0.17$). No correlation was observed between NO and TNF-alpha in PBMNCs from T2DM patients and ND controls.

3.3. The Plasma MDA and Proinflammatory Cytokine (IL-6 and TNF-Alpha) Concentrations Are Elevated in T2DM. Table 2 shows that T2DM patients had enhanced plasma concentrations of MDA, IL-6, and TNF-alpha compared with ND ($P < 0.05$). No difference was observed in NO levels between T2DM patients and ND ($P > 0.05$). The results, expressed as the means ± SD, were MDA, 14.5 ± 3.5 and 8.7 ± 3.3; IL-6, 119.1 ± 23.3 and 97.6 ± 13.5; TNF-alpha, 78.7 ± 32.7 and 58.5 ± 29.5; NO, 53.5 ± 12.9 and 51.13 ± 8.7, for T2DM patients and ND controls, respectively.

3.4. Plasmatic Nitric Oxide Levels Correlate with MDA and IL-6 Levels in the Plasma from T2DM Patients. Correlations between the levels of NO and IL-6 and TNF-alpha and MDA are shown in Figure 3. Strong positive correlation was observed between NO and IL-6 in T2DM patients ($r = 0.72$, $P < 0.0001$). The results also demonstrated a significantly negative correlation between NO and MDA in T2DM patients ($r = -0.47$, $P = 0.0093$).

3.5. Plasmatic Triglyceride Levels in T2DM Patients Correlate with the Plasma Levels of MDA, IL-6, and TNF-Alpha. Figure 4 shows the Pearson's correlations between the levels of triglyceride and NO and IL-6 and TNF-alpha in the plasma from T2DM patients and ND. The triglyceride levels were positively correlated with MDA ($r = 0.43$, $P = 0.018$), IL-6 ($r = 0.52$, $P = 0.003$), and TNF-alpha ($r = 0.37$, $P = 0.048$) in the plasma of T2DM patients.

3.6. Plasmatic Glucose Levels in T2DM Patients Correlate with the Plasma Levels of Triglycerides, MDA, IL-6, and TNF-Alpha. Figure 5 shows the Pearson's correlations between the levels of glucose and triglycerides, NO, MDA, and proinflammatory cytokines levels in the plasma from T2DM patients and ND controls. The glucose levels were positively correlated with triglycerides ($r = 0.40$, $P = 0.03$), MDA ($r = 0.60$, $P = 0.0006$), IL-6 ($r = 0.40$, $P = 0.04$), and TNF-alpha ($r = 0.35$, $P = 0.05$) in the plasma of T2DM patients.

Table 2: Plasma levels of oxidative stress biomarkers and proinflammatory cytokines.

Parameter	T2DM ($n = 29$)	ND ($n = 16$)	P
Nitric Oxide (μM)	53.5 ± 12.9	51.13 ± 8.7	ns
MDA (μM)	14.5 ± 3.5	8.7 ± 3.3	<0.05
IL-6 (pg/mL)	119.1 ± 23.3	97.6 ± 13.5	<0.05
TNF-alpha (pg/mL)	78.7 ± 32.7	58.5 ± 29.5	<0.05

Data as means ± SD.
ns: not significant.
Significant differences between the groups were determined using Student's t-test ($P < 0.05$).

4. Discussion

The results obtained in the present study showed that hyperglycemia in diabetes primes PBMNCs *in vivo*, inducing the *in vitro* upregulation of NO and proinflammatory cytokines in cells stimulated with palmitate. The plasmitic evaluation demonstrated greater levels of triglycerides, MDA, IL-6, and TNF-alpha in T2DM patients compared with ND. No difference was observed in the NO plasma levels between T2DM patients and ND. In addition, the results of this study revealed that the levels of NO were correlated with MDA and IL-6, and levels of triglycerides were correlated with MDA, IL-6, and TNF-alpha in the plasma from T2DM patients.

Diabetes is a multifactorial disease characterized by hyperglycemia and hyperlipidemia, which are important risk factors for endothelial dysfunction resulting in cardiovascular events [49]. FFAs, particularly SFA, have been shown to induce a proinflammatory profile associated with obesity, T2DM, insulin resistance, and dyslipidemia [4, 8–11]. The results presented herein show the inflammatory effects of the saturated fatty acid palmitate on PBMNCs from T2DM patients but not in cells from ND (Figure 1), suggesting that hyperglycemia plays a role in palmitate-induced inflammation. Studies have shown that the combined effect of high glucose and FFA levels in human monocytes modulate macrophage proliferation involving glucose-dependent oxidation of LDL, potentiate cytotoxic effects via superoxide overproduction, and amplify inflammation via TLR [21, 50, 51]. However, Tripathy et al. [32] demonstrated that an increase in FFA concentration induces oxidative stress and inflammation in human leukocytes from ND subjects. These discrepancies might be associated with differences in the experimental protocols.

The inflammatory changes observed in the presence of palmitate could be associated with NF-kappaB activation [21, 28, 32, 52–55]. NF-kappaB is a key mediator that regulates immune and inflammatory responses and modulates multiple proinflammatory target genes in endothelial cells, vascular smooth muscle cells, and macrophages [56]. The activation of NF-kappaB leads to the increased production of adhesion molecules, leukocyte-attracting chemokines, various inflammatory cytokines, including TNF-alpha and IL-6, and NO through iNOS expression [57–60].

NO has anti- or proinflammatory properties [61]. NO plays an important role in vascular homeostasis, and in

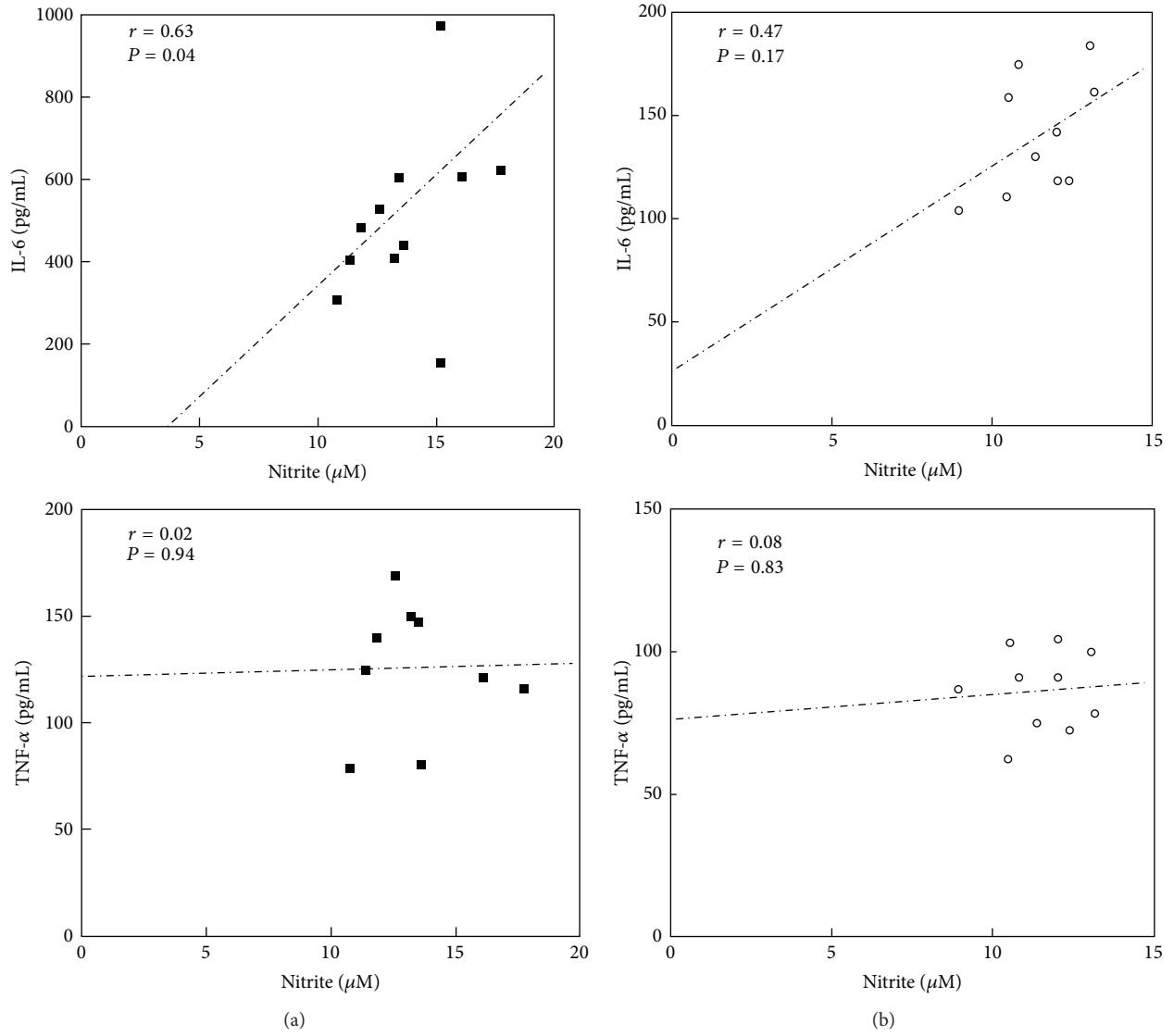

Figure 2: Pearson's correlation coefficients between NO and proinflammatory cytokines in PMBNCs from T2DM patients (a) and nondiabetic controls (b) after stimulation with palmitate. $n = 10$ for each group.

immune cells, NO regulates antimicrobial and antitumor activities, although excess NO production might cause tissue damage and is associated with acute and chronic inflammation [56, 62]. Nitric oxide synthase (NOS) synthesizes NO from L-arginine using NADPH and oxygen as cosubstrates [63]. Three isoforms of NO synthase have been described: neuronal (nNOS or NOS 1), inducible (iNOS or NOS 2), and endothelial (eNOS or NOS 3) [64]. Activated macrophages and neutrophils produce large amounts of NO through iNOS activity [65, 66]. The results of this study demonstrated increased NO production and a positive correlation between NO and IL-6 levels in palmitate-stimulated PBMNCs from T2DM patients, suggesting that iNOS expression can be elevated through palmitate-induced proinflammatory cytokine secretion. No differences were observed in the cells from ND controls (Figures 1 and 2). Unbound palmitic acid treatment increased NO production in skeletal muscle [67]. However, in endothelial cells, FFA induced the inhibition of eNOS, thereby attenuating NO production [68–71].

To evaluate *in vivo* inflammation, we quantified the plasma levels of NO, the oxidative stress biomarker (MDA), and proinflammatory cytokines (IL-6 and TNF-alpha) in T2DM patients and ND controls. Consistent with other studies [72–93], the results of the present study demonstrated elevated levels of IL-6 and TNF-alpha, reflecting the activation of innate immune cells, and high levels of MDA, indicating the presence of oxidative stress in T2DM patients compared with ND controls. Diabetic conditions (hyperglycemia and hyperlipidemia) increase proinflammatory and oxidative stress levels, culminating in endothelium dysfunction [1, 27, 42, 56, 90, 94, 95]. Oxidative stress reduced NO production through eNOS [56], and the increased levels of superoxide could react with NO to produce peroxynitrite, a highly toxic product [23, 96]. Peroxynitrite nitrates

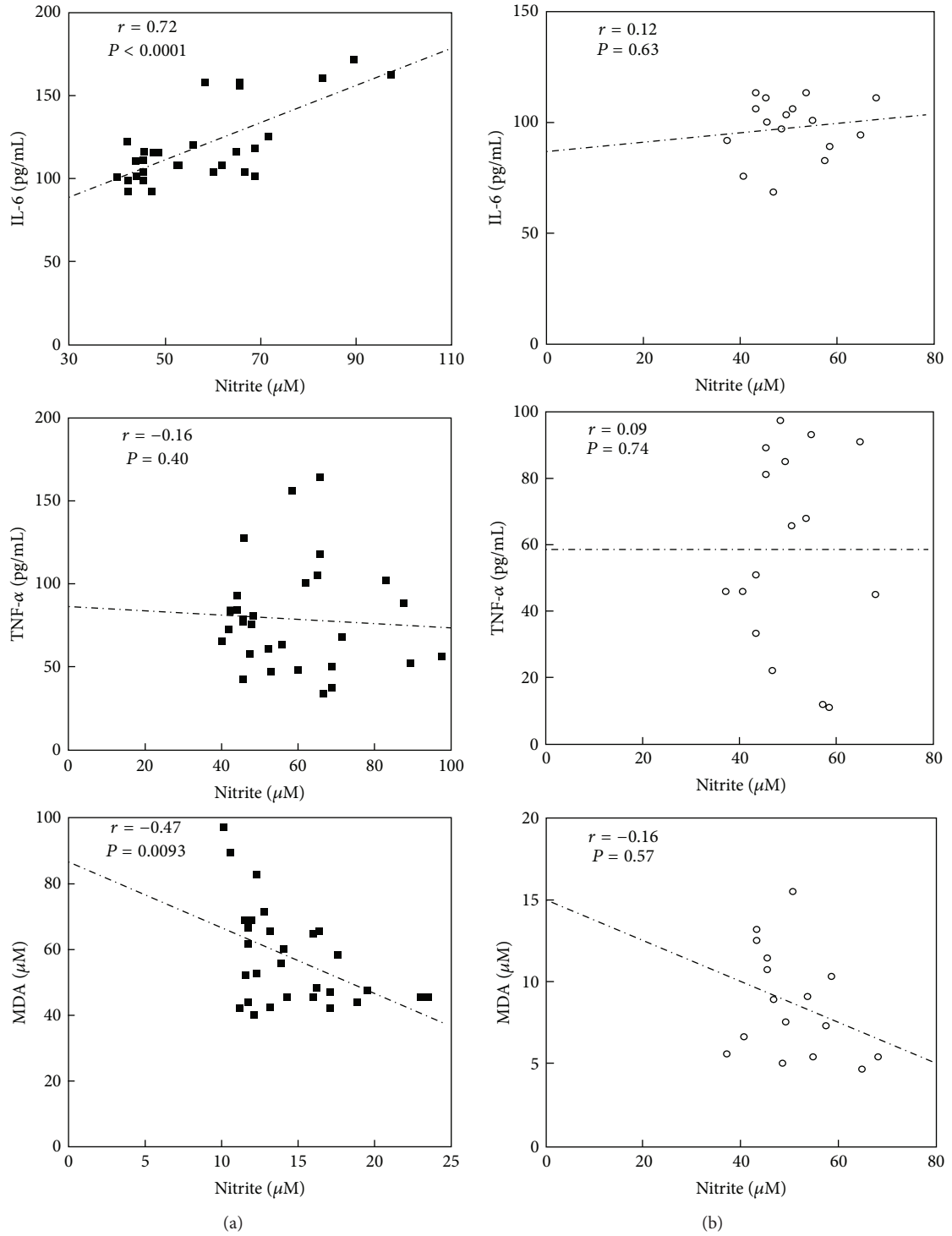

Figure 3: Pearson's correlation coefficients between nitric oxide and proinflammatory cytokines and MDA in the plasma from T2DM patients (a) and nondiabetic controls (b). $n = 29$ for T2DM patients and 16 for nondiabetic controls.

the tyrosine residues in a number of proteins and modulates their functions [97, 98]. The results in the present study did not show any differences in the plasma NO levels between the studied groups (Table 2). However, we observed a negative association between NO and MDA levels in the plasma from T2DM patients, suggesting that increased oxidative stress could affect NO biodisponibility, leading to endothelial dysfunction in diabetes (Figure 3).

The results obtained in the present study also demonstrated high levels of triglycerides in the plasma from T2DM patients compared with ND controls (Table 1). FFAs are stored in the body in the form of triglycerides and are released

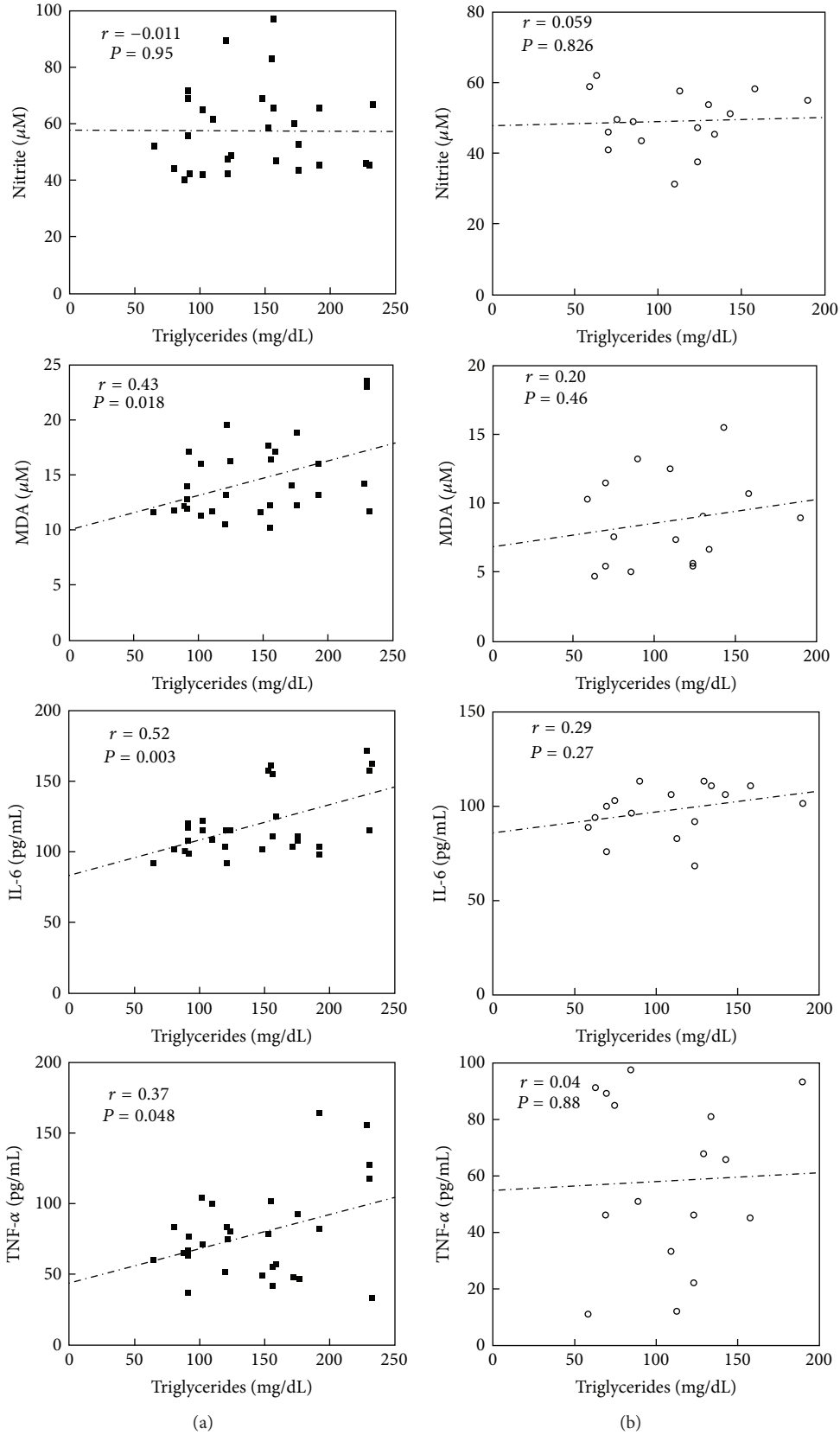

Figure 4: Pearson's correlation coefficients between triglycerides and proinflammatory cytokines and MDA in the plasma of T2DM patients (a) and nondiabetic controls (b). $n = 29$ for T2DM patients and 16 for nondiabetic controls.

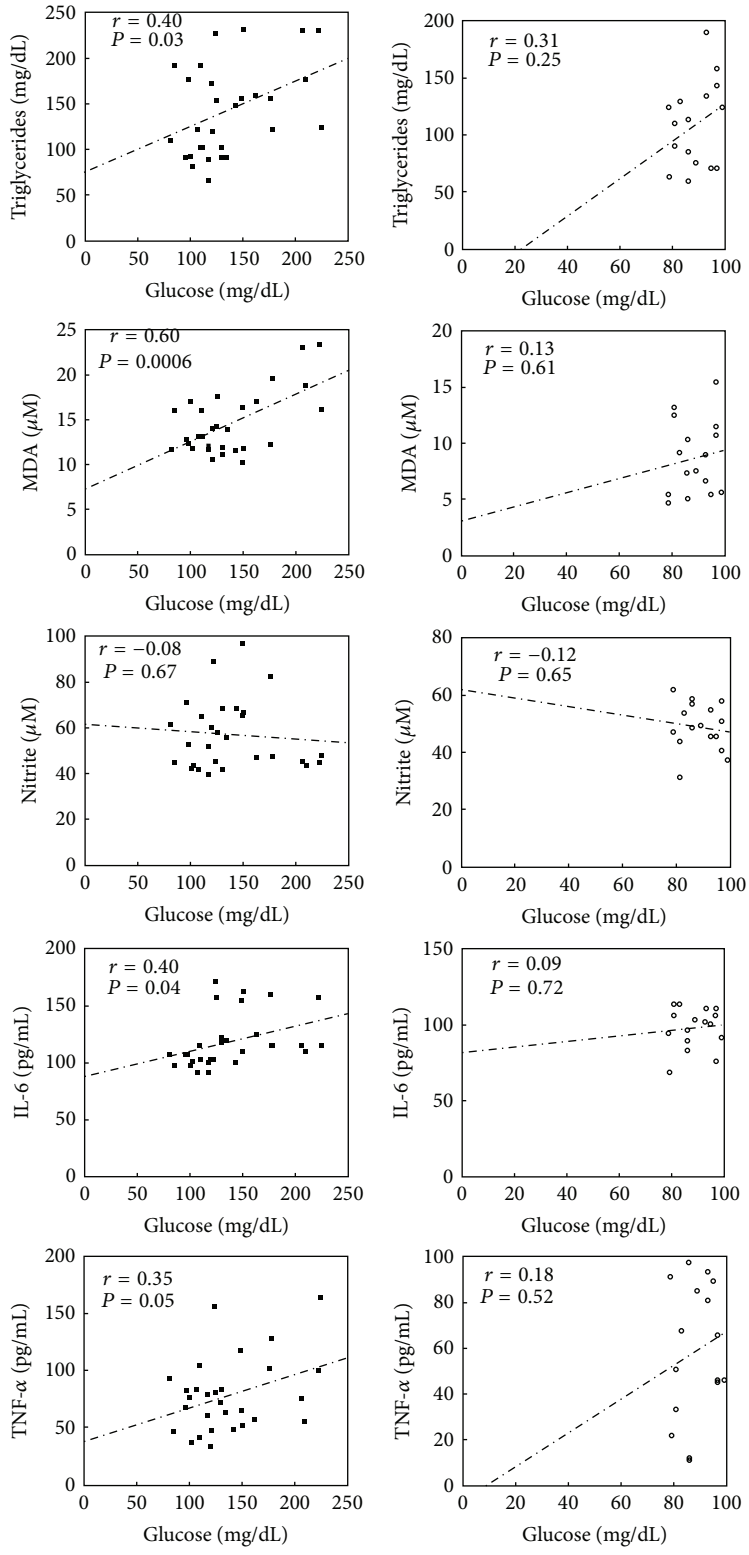

Figure 5: Pearson's correlation coefficients between glucose and triglycerides, MDA, nitric oxide, and proinflammatory cytokines in plasma of T2DM (a) patients and nondiabetic controls (b). $n = 29$ for T2DM and 16 for nondiabetic controls.

into tissues through lipolysis, a process regulated through insulin [99]. Impaired insulin signaling increases lipolysis, resulting in increased FFA levels [100, 101]. The results of the present study showed that triglycerides levels are positively associated with the MDA, IL-6, and TNF-alpha levels in the plasma from T2DM patients, but this correlation was not observed in the plasma from ND controls. No correlation was observed between triglycerides and NO in the plasma from the studied groups (Figure 4). Glucose levels are positively correlated with the triglycerides, MDA, IL-6, and TNF-alpha levels in the plasma from T2DM patients, but not in the plasma from ND controls (Figure 5).

Accumulating evidence has shown that the regulation of dyslipidemia is of equal importance for the regulation of hyperglycemia and hypertension in the care of patients with T2DM. Hyperlipidemia represents a major risk factor for the development of vascular dysfunction and atherosclerosis [27–30, 42, 43]. Most T2DM patients are obese and have elevated plasma FFA levels [102, 103]. Moreover, high-fat diets might induce metabolic dysfunction and inflammation through the release of FFA through lipolysis and proinflammatory cytokines through downstream signaling [104, 105].

FFAs have been suggested to induce chronic low-grade inflammation, activate the innate immune system, and cause deleterious effects on vascular cells and other tissues through inflammatory processes. The results obtained in the present study demonstrated that hyperglycemia in diabetes exacerbates *in vitro* inflammatory responses in PBMNCs stimulated with high levels of SFA (palmitate). Furthermore, the results suggest that the endothelium levels of NO could be regulated through oxidative stress and high levels of triglycerides are correlated with oxidative stress and proinflammatory cytokine secretion in T2DM patients. Endothelial dysfunction is associated with several pathophysiological conditions in diabetes [56]. Combined therapy targeting the intracellular mechanisms underlying metabolic alterations leading to endothelial dysfunction is an important issue in the prevention of vascular complications associated with diabetes. The simultaneous control of hyperglycemia and hypertriglyceridemia is necessary to ameliorate the progression to diabetic vasculopathy.

Conflict of Interests

The authors confirm that there is no conflict of interests.

Acknowledgments

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Research Article

Effects of Paracetamol on NOS, COX, and CYP Activity and on Oxidative Stress in Healthy Male Subjects, Rat Hepatocytes, and Recombinant NOS

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Paracetamol (acetaminophen) is a widely used analgesic drug. It interacts with various enzyme families including cytochrome P450 (CYP), cyclooxygenase (COX), and nitric oxide synthase (NOS), and this interplay may produce reactive oxygen species (ROS). We investigated the effects of paracetamol on prostacyclin, thromboxane, nitric oxide (NO), and oxidative stress in four male subjects who received a single 3 g oral dose of paracetamol. Thromboxane and prostacyclin synthesis was assessed by measuring their major urinary metabolites 2,3-dinor-thromboxane B_2 and 2,3-dinor-6-ketoprostaglandin $F_{1\alpha}$, respectively. Endothelial NO synthesis was assessed by measuring nitrite in plasma. Urinary 15(*S*)-8-*iso*-prostaglanding $F_{2\alpha}$ was measured to assess oxidative stress. Plasma oleic acid oxide (*cis*-EpOA) was measured as a marker of cytochrome P450 activity. Upon paracetamol administration, prostacyclin synthesis was strongly inhibited, while NO synthesis increased and thromboxane synthesis remained almost unchanged. Paracetamol may shift the COX-dependent vasodilatation/vasoconstriction balance at the cost of vasodilatation. This effect may be antagonized by increasing endothelial NO synthesis. High-dosed paracetamol did not increase oxidative stress. At pharmacologically relevant concentrations, paracetamol did not affect NO synthesis/bioavailability by recombinant human endothelial NOS or inducible NOS in rat hepatocytes. We conclude that paracetamol does not increase oxidative stress in humans.

1. Introduction

Nitric oxide (NO), prostaglandin (PG) I_2, that is, prostacyclin (PGI_2), and thromboxane A_2 (TxA_2) are important short-lived signaling molecules involved in many physiological and pathological processes. Thus, PGI_2 and NO are potent vasodilators and inhibitors of platelet aggregation. Contrarily, TxA_2 is a strong vasoconstrictor and inductor of platelet aggregation. NO is synthesized from L-arginine (Arg) by constitutive and inducible NO synthase (NOS) isoforms. Prostaglandin H synthase (PGHS) isoforms, generally termed cyclooxygenase (COX), convert arachidonic acid (AA) to the collectively named prostanoids. The L-arginine/NO pathway is generally accepted to interact with the COX pathway and to modulate its activity [1–4]. For instance, the inducible NOS (iNOS) isoform has been shown to bind to the inducible COX isoform (COX-2) and to *S*-nitrosylate and activate COX-2 [2]. The role of NO in prostaglandin biology has been recently updated by Kim [4]. Potential mechanisms of direct NOS-COX cross-talk may include (1) binding of NO to the iron atom of the heme group of COX, (2) reaction of the nitrosyl cation (NO^+) with sulfhydryl (SH) groups of cysteine (Cys) moieties of COX to form *S*-nitroso-COX, and (3) reaction of peroxynitrite ($ONOO^-$), that is, the reaction product of NO radical ($^\bullet NO$) and superoxide radical anion ($O_2^{\bullet -}$) produced either by NOS itself or by other enzymes including COX and CYP [3], with SH groups of Cys residues or with tyrosine (Tyr) residues of COX being involved in the catalytic process [2]. *S*-Nitrosylation of COX-Cys moieties by higher oxides of NO, notably dinitrogen trioxide (N_2O_3), and

by $ONOO^-$ and *S*-transnitrosylation of COX-Cys moieties by low-molecular-mass *S*-nitrosothiols have been shown to both enhance and inhibit COX activity. Nitration of Tyr residues located in the catalytic domain of COX is assumed to inhibit COX activity [2, 4–6]. On the other hand, $ONOO^-$ has been reported to enhance COX activity presumably by increasing the peroxide concentration that is required for the peroxidase activity of COX [7].

Paracetamol (acetaminophen, APAP) is one of the most frequently applied drugs worldwide and is considered generally a safe analgesic and antipyretic drug in therapeutic dosage, which lacks however appreciable anti-inflammatory and antiplatelet activity [10]. The mechanism of the analgesic and antipyretic effects of paracetamol is not fully established, yet inhibition of PGHS activity by paracetamol in different cell and tissue types is generally assumed to be the main mode of paracetamol's analgesic and antipyretic action. PGHS possesses both peroxidase and cyclooxygenase activity. Paracetamol is believed to inhibit the peroxidase catalytic site of PGHS, unlike the majority of nonsteroidal anti-inflammatory drugs (NSAIDs) and the PGHS2 inhibitors. In vitro, paracetamol is a much stronger inhibitor of prostanoid synthesis in endothelial cells than in platelets. In particular, paracetamol is a weak inhibitor of TxA_2 synthesis in platelets. It is also remarkable that the inhibitory potency of paracetamol is inversely correlated with the PGHS concentration (for a review, see [10]). These particular characteristics distinguish paracetamol from NSAIDs including acetylsalicylic acid (ASA).

In vitro, PGHS activity can be assessed by measuring the production rate of various primary prostanoids, such as PGE_2, PGI_2, and TxA_2. Because of the remarkable chemical instability of PGI_2 and TxA_2, their stable hydrolysis products, that is, 6-keto-$PGF_{1\alpha}$ and TxB_2, respectively, are measured instead of PGI_2 and TxA_2 [11]. In vivo, measurement of PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2 in plasma is associated with artefactual prostanoid synthesis during blood sampling and may lead to incorrect conclusions with regard to PGHS activity [12]. This especially applies to TxA_2 which is produced in high amounts in activated platelets [13]. Measurement of PGE_2 in the urine reflects renal PGE_2 synthesis. By far more reliable is the measurement of major urinary metabolites of prostanoids, such as 2,3-dinor-TxB_2 for TxA_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$ for PGI_2, and the major urinary metabolite of PGE_2 (PGE-MUM) for systemic PGE_2 production [11]. This can be best accomplished by means of analytical technologies which have high inherent sensitivity and selectivity such as gas chromatography-mass spectrometry (GC-MS) and more so gas chromatography-tandem mass spectrometry (GC-MS/MS) (for a review, see [11]).

Recently, Sudano and colleagues [9] reported that paracetamol (1 g TID for 2 weeks on top of standard cardiovascular therapy) increased ambulatory mean systolic and diastolic blood pressure by about 3 and 2 mmHg, respectively, without changing endothelium and platelet function in patients with coronary artery disease (CAD). Sudano et al. [9] concluded that, particularly in patients at increased cardiovascular risk, use of paracetamol should be evaluated as rigorously as traditional NSAIDs and selective COX2 inhibitors. In that study, plasma and urine PGE_2 as well as plasma TxB_2 did not change upon paracetamol administration [9]. However, as mentioned above, measurement of PGE_2 and TxB_2 in plasma is prone to artefactual prostanoid synthesis [12, 13], whereas measurement of PGE_2 in the urine does not provide information about PGHS-catalyzed synthesis of the two antagonists TxA_2 and PGI_2 [11].

In humans, oral administration of paracetamol (500 mg) has been reported not to result in decreased excretion rate of 2,3-dinor-TxB_2, unlike aspirin (500 mg) or indomethacin (50 mg), as measured by GC-MS [14]. Also, in contrast to aspirin (3 g for 2 days), oral administration of paracetamol (3 g for 2 days) has been reported not to reduce urinary excretion of PGE_2 but to weakly reduce PGE-MUM excretion indicating inhibition of systemic PGE_2 synthesis [15]. On the other hand, a single oral dose of 500 mg paracetamol has been shown to reduce urinary excretion rate of 2,3-dinor-6-keto-$PGF_{1\alpha}$ for 6–8 h by maximally 60% (i.e., inhibition of PGI_2 synthesis), without reducing urinary excretion rate of 2,3-dinor-TxB_2 (i.e., no inhibition of TxA_2 synthesis) [16]. The results of these in vivo studies in human subjects suggest that orally administered paracetamol, at a single dose of 500 mg or at a cumulative dose of 3000 mg per day, does not inhibit remarkably TxA_2 synthesis, but it may temporarily inhibit PGI_2 synthesis.

The ramifications between NOS and COX pathways have been frequently investigated in the past (reviewed in [4]), but results are inconsistent. For instance, in murine macrophages, paracetamol, at pharmacologically relevant plasma concentrations (60–120 μM), has been reported not to affect iNOS activity [17]. At suprapharmacological concentrations (2, 5, and 10 mM), paracetamol has been reported to inhibit iNOS gene expression and iNOS activity in RAW 264.7 cell line macrophages [18]. By contrast, paracetamol (up to 10 mM) has been reported not to affect neuronal NOS (nNOS) and iNOS activity in rat cerebellum and HUVECs [19]. Others have reported that paracetamol (100 μM) did not affect nNOS activity in cerebellum but inhibited NOS activity in murine spinal cord slices as measured by the radiolabelled L-citrulline assay [20]. The effect of paracetamol on in vivo in humans is elusive.

Because paracetamol, when applied at pharmacological doses, inhibits the synthesis of the vasodilatatory and antiaggregatory PGI_2 much stronger and sustainably than the synthesis of the vasoconstrictory and thrombogenic TxA_2 in humans, we wondered whether the paracetamol-induced shifting of the balance between vasodilatatory/antiaggregatory and vasoconstrictory/thrombogenic COX-related homeostasis may induce processes that lead to enhanced synthesis of the vasodilatatory/antiaggregatory NO, thus counteracting blood pressure fall and platelet activation. Preliminary investigations of our group showed that paracetamol, administered in therapeutic doses to healthy humans (up to 10 mg/kg), did not change whole body NO synthesis (data not shown), suggesting that a potential effect of paracetamol on NOS activity is likely to require much higher, suprapharmacological doses of this drug. In consideration of the toxicological potency of high paracetamol doses, we

investigated the effects of a single oral 3 g dose in four healthy volunteers. To our knowledge, the effects of such a high single oral dose of paracetamol on PGI_2, TxA_2, and NO synthesis in humans have not been investigated so far. Because of the high dose used in the human study, paracetamol may induce oxidative stress and decrease NO bioavailability [21]. We therefore measured the oxidative stress biomarker 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [22] in plasma and urine. Nitrite in plasma was measured as a biomarker of NO synthesis and bioavailability (reviewed in [23]). In addition, we performed in vitro studies on recombinant endothelial NOS (eNOS) and inducible NOS (iNOS) in rat hepatocytes to test potential effects of paracetamol on NO synthesis and bioavailability.

2. Materials and Methods

2.1. Subjects and Study Performance. Four healthy nonsmoking male adults (aged 39, 40, 44, and 64 years) participated in the study and gave their informed consent to the study. The volunteers received orally six 500 mg paracetamol tablets (Ratiopharm) at once. Dosage was each 29 mg/kg for volunteer A and volunteer B, 37 mg/kg for volunteer C, and 52 mg/kg for volunteer D. Volunteers were not fasting but they did not eat in the first three hours following paracetamol administration. Before and after paracetamol administration, venous blood and urine were collected in 30 and 60 min intervals over an observation period of 6 h for analysis of biochemical parameters as described below. Venous blood (8 mL) was drawn by using 9 mL EDTA vacutainers (Sarstedt, Germany) and centrifuged immediately (800 ×g, 4°C, 5 min). Plasma was decanted, portioned in 0.1 and 1.0 mL aliquots as required for each biochemical parameter, and stored frozen at −80°C until analysis. Urine from spontaneous micturition was collected in 45 mL polypropylene tubes, aliquoted in 0.1 and 1.0 mL portions according to the requirement of the individual biochemical parameters, and stored at −20°C until analysis.

2.2. Analysis of Biochemical Parameters in the Human Study. All samples of this study were analyzed within 10 days after collection. In GC-MS and GC-MS/MS methods, stable-isotope labelled analogs were used as internal standards as reported in the respective references cited below. We found that paracetamol added to pooled human plasma at concentrations of 10, 25, 50, 75, and 100 mg/L did not interfere with the analysis of the biochemical parameters measured in the study plasma samples (data not shown). Data from this study are reported as mean ± standard error of the mean (SEM).

2.2.1. Measurement of Paracetamol. Plasma paracetamol concentration was determined by reverse phase HPLC (250 × 4 mm i.d., 5 μm particle size) with isocratic elution (mobile phase: 45 mM ammonium sulphate-acetonitrile, 10 : 1, v/v; flow rate: 1 mL/min) with UV absorbance detection at 236 nm.

2.2.2. Measurement of Prostanoids and Creatinine. PGI_2 and TxA_2 synthesis was assessed by GC-MS/MS by measuring in 1 mL urine aliquots the respective major urinary metabolites [11], that is, 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TxB_2, exactly as described elsewhere [24]. PGE_2 and free nonconjugated 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in urine (1 mL) and free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in plasma (1 mL) were measured by GC-MS/MS after extraction by immunoaffinity column chromatography as described previously [25]. Urinary excretion rate of the eicosanoids was corrected for creatinine excretion [11] and is expressed in nmol prostanoid/mol creatinine. Urine creatinine was measured in 10 μL urine aliquots by GC-MS as reported elsewhere [26].

2.2.3. Analysis of the L-Arginine/NO Pathway. Nitrite and nitrate were measured simultaneously in 100 μL aliquots of plasma or urine by GC-MS as described elsewhere [27]. Urinary excretion rate of nitrite and nitrate was corrected for creatinine excretion as well. Arginine and the endogenous NOS activity inhibitor asymmetric dimethylarginine (ADMA) were measured by GC-MS and GC-MS/MS, respectively, in 100 μL aliquots of ultrafiltrate obtained from plasma by centrifugation according to previously reported procedures [8].

2.2.4. Additional Analyses. Total homocysteine (hCys) in plasma (0.1 mL) was measured by a commercially available fluorescence polarimetry immunoassay (FPIA). In vivo CYP activity [28] was assessed by measuring oleic acid oxide (*cis*-EpOA) in 1 mL aliquots of plasma as described elsewhere [29].

2.2.5. Quality Control. Quality control (QC) samples were analyzed alongside study samples for all biochemical parameters. Accuracy and precision in the QC samples were within generally accepted ranges; that is, bias and imprecision levels were below 20%.

2.3. Effect of Paracetamol on Recombinant Human eNOS Activity. The effect of paracetamol on NOS activity in vitro was investigated by using a commercially available (ALEXIS, Grünberg, Germany) recombinant human endothelial NOS (heNOS) and by measuring simultaneously formation of [^{15}N]nitrite and [^{15}N]nitrate from L-[*guanidine*-$^{15}N_2$]arginine by means of a GC-MS assay [30]. Incubations were performed at 37°C in 50 mM potassium phosphate buffer (1000 μL, pH 7) containing heNOS (50 μg/mL), L-[*guanidine*-$^{15}N_2$]-arginine (20 μM, Cambridge Isotope Labs, Andover, MA, USA), and all NOS cofactors (all purchased from Sigma-Aldrich, Steinheim, Germany) and prosthetic groups (10 μM tetrahydrobiopterin, 800 μM NADPH, 5 μM FAD, 5 μM for FMN, 500 nM calmodulin, and 500 μM $CaCl_2$ (Merck, Darmstadt, Germany)). Reactions were terminated by addition of 400 μL aliquots of ice cold acetone and samples were processed for GC-MS analysis of [^{15}N]nitrite and [^{15}N]nitrate. Unlabeled nitrite and nitrate were used as internal standards for [^{15}N]nitrite and [^{15}N]nitrate,

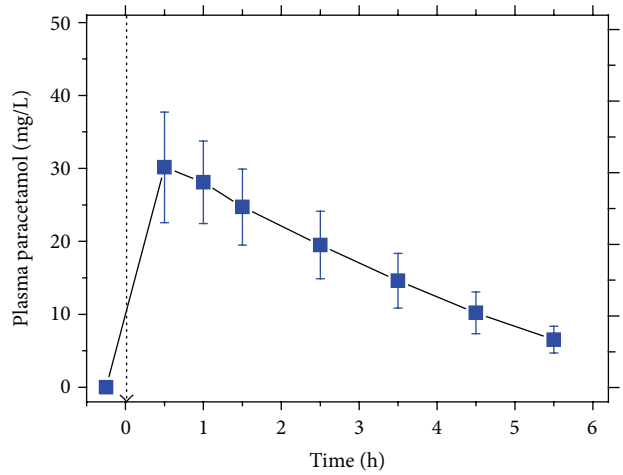

Figure 1: Plasma concentration of paracetamol before and after administration (time zero is indicated by the dashed arrow) of a single oral 3 g dose of paracetamol to four male subjects. Data are shown as mean ± SEM.

respectively. Data are shown as mean ± standard deviation (SD) from two independent experiments.

2.4. Effect of Paracetamol on iNOS Activity in Cultured Rat Hepatocytes Proliferating In Vitro. The effect of paracetamol on iNOS activity was investigated in primary rat hepatocytes proliferating in vitro as described recently by measuring formation of [^{15}N]nitrite and [^{15}N]nitrate by GC-MS [31]. In some experiments, LiCl (10 mM) was used to enhance expression of iNOS-mRNA and cell growth [31]. Incubations were performed at 37°C in the presence of 5 mM L-[*guanidine*-$^{15}N_2$]-arginine added at the time -22 h. Reactions were terminated by addition of 400 μL aliquots of ice cold acetone, and samples were processed for GC-MS analysis of [^{15}N]nitrite and [^{15}N]nitrate. Data are shown as mean ± SD from three independent experiments.

2.5. Statistical Analysis. Because of considerable differences in the baseline concentrations of some of the biochemical parameters measured in the four subjects, changes and statistical significance were calculated by setting the respective baseline levels to 100%. Statistical significance ($P < 0.05$) was evaluated by using unpaired t-test and comparing the data obtained at various times to the baseline values or to the 0.5 h values when percentage changes were compared.

3. Results

3.1. Effect of High-Dose Paracetamol on COX, NOS, and CYP Activity and on Oxidative Stress in Humans. Mean maximum paracetamol plasma concentration (C_{max}) was 30.2 mg/L (200 μmol/L) (Figure 1). This value is consistent with a C_{max} value of about 20 mg/L that has been reached after oral administration of 2000 mg of paracetamol [32]. In the urine samples, we measured by reverse phase HPLC with UV absorbance detection comparable creatinine-corrected excretion rates of the paracetamol glucuronide and sulphate metabolites (data not shown).

Upon 3 g paracetamol intake, considerable and sustained decrease in creatinine-corrected 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion rate was seen, suggesting strong PGI_2 inhibition by paracetamol (Figure 2(a)). Maximum and statistically significant PGI_2 inhibition of about 60 to 70% was reached 1 h, 1.5 h, and 2.5 h after paracetamol administration. The extent of the decrease seen in the 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion rate in the present study is comparable to that seen upon administration of a 500 mg oral paracetamol dose [14]. Paracetamol caused only a moderate, statistically insignificant decrease in 2,3-dinor-TxB_2 excretion in the four volunteers (Figure 2(b)). In three out of the four volunteers, maximum TxA_2 inhibition of about 70% was reached 1.5 h after administration, but the duration of TxA_2 inhibition was relatively short (not shown). Figure 2(c) shows that the PGI_2/TxA_2 molar ratio decreased by a factor of 2 to 3 upon paracetamol administration, although statistical significance failed by a hair 1.5 h ($P = 0.069$) and 2.5 h ($P = 0.056$) after paracetamol ingestion. Paracetamol seemed to decrease very weakly the excretion of PGE_2 in the urine (Figure 2(d)), suggesting that even 3 g of paracetamol taken at once is not able to inhibit renal synthesis of PGE_2 in the four volunteers enrolled in the study.

Paracetamol-induced changes in systemic prostacyclin synthesis were not accompanied by noteworthy changes in the plasma concentration of total hCys (Figure 3(a)) or in the urinary excretion rate of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (Figure 3(b)), suggesting no elevation or reduction of oxidative stress upon high-dosed paracetamol administration.

Because of the considerable difference in the baseline plasma nitrite and nitrate concentrations measured in the four subjects, changes in plasma nitrite and nitrate were calculated and presented as percentage of the respective baseline levels. Figure 4(a) shows moderate increases in plasma nitrite concentration which were statistically significantly higher when the 2.5 and 3.5 h values were compared with the 0.5 h values. Changes in plasma nitrate concentrations (Figure 4(b)) and urine nitrite (Figure 4(c)) and urine nitrate (Figure 4(d)) excretion were not statistically significantly different. Finally, plasma Arg and ADMA concentrations did not change upon paracetamol ingestion (Figure 4(e)).

Previously, we showed that the whole body activity of CYP isoforms can be assessed by measuring the concentration of the free, that is, nonesterified, oleic acid oxide *cis*-EpOA in plasma [28]. Figure 5 shows an abrupt increase in mean plasma *cis*-EpOA concentration 2.5 h after paracetamol administration followed by an abrupt fall to baseline level 1 h later. This finding may suggest a very short-term paracetamol-induced elevation of CYP activity. However, we also found a very similar change in the plasma concentration of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (Figure 5). Previously, we observed that addition of phospholipase A_2 (PLA_2) to human serum increased in parallel the concentration of both free *cis*-EpOA and free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [25]. Therefore, the temporary short-time increases in *cis*-EpOA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ seen 2.5 h after paracetamol administration may have

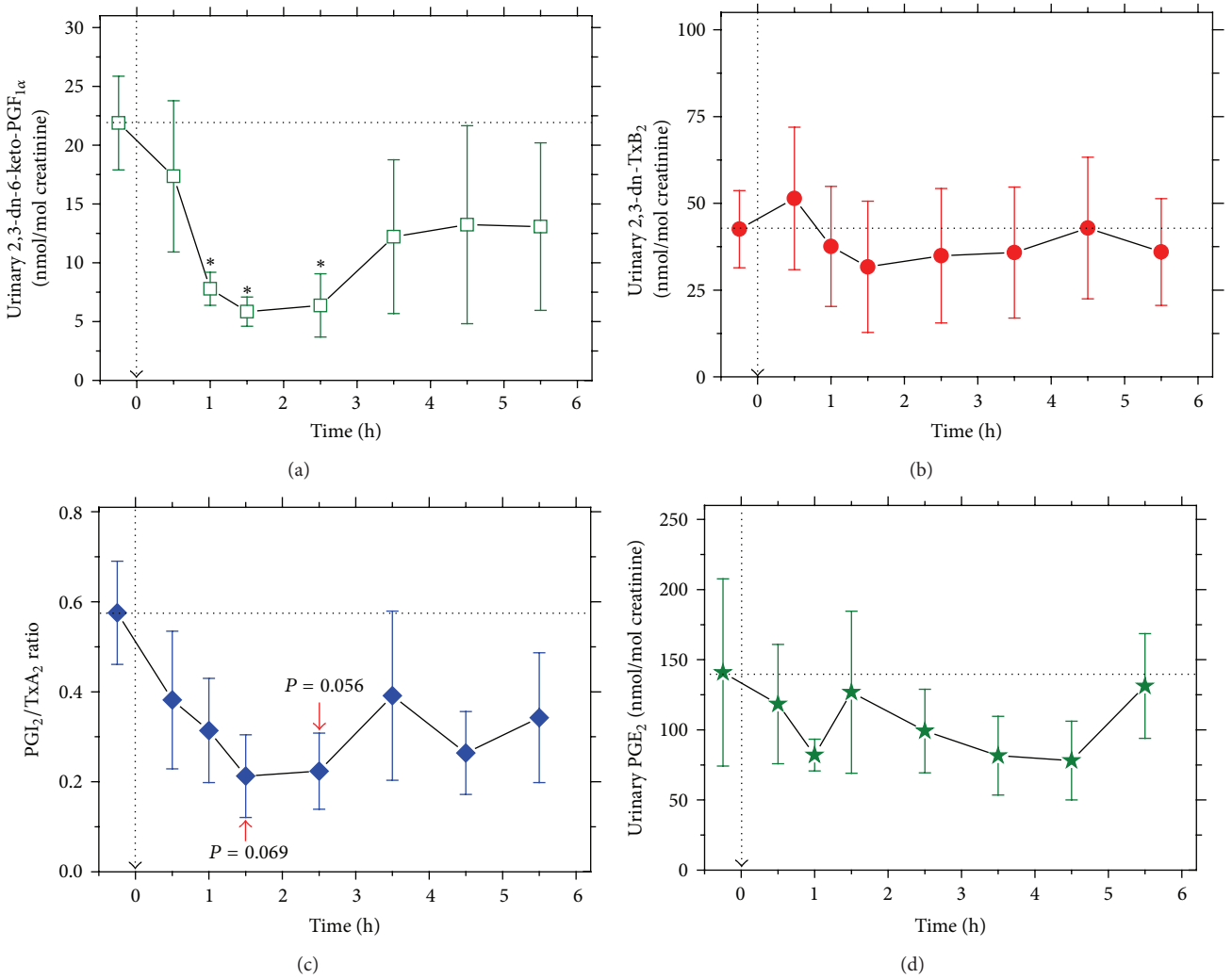

FIGURE 2: Effect of a single oral 3 g dose of paracetamol on systemic prostacyclin and thromboxane synthesis and on renal synthesis of PGE_2 in four healthy volunteers (time zero and baseline values are indicated by dashed arrows). (a) Creatinine-corrected urinary excretion of 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (2,3-dn-6k-$PGF_{1\alpha}$) as a measure of systemic PGI_2 synthesis. (b) Creatinine-corrected urinary excretion of 2,3-dinor-thromboxane B_2 (2,3-dn-TxB_2) as a measure of systemic TxA_2 synthesis. (c) PGI_2/TxA_2 molar ratio calculated from the 2,3-dn-6k-$PGF_{1\alpha}$ and 2,3-dn-TxB_2 excretion rates shown in (a) and (b), respectively. (d) Creatinine-corrected urinary excretion of PGE_2 as a measure of renal PGE_2 synthesis. An asterisk in (a) indicates statistical significance ($P < 0.05$) compared to basal values. Data are shown as mean ± SEM.

resulted from release of presumably hepatic PLA_2 into the blood.

3.2. Effects of Paracetamol on Recombinant heNOS Activity and iNOS in Rat Hepatocytes. At the therapeutically relevant concentration of 100 μM (i.e., 15 mg/L), paracetamol had only a weak effect on the formation of [^{15}N]nitrite and [^{15}N]nitrate in incubation mixtures of a recombinant heNOS (Figure 6). Linear regression of analysis between the concentrations of [^{15}N]nitrite and [^{15}N]nitrate in the presence of paracetamol versus the concentrations of [^{15}N]nitrite and [^{15}N]nitrate in the absence of paracetamol revealed a slope value of 0.834 (Figure 6(b)). This finding suggests that paracetamol inhibited heNOS-catalyzed ^{15}NO formation (i.e., [^{15}N]nitrite and [^{15}N]nitrate) from L-[*guanidine*-$^{15}N_2$]-arginine in average by 16.6%, notably for incubation times longer than 10 min. Similar small effects of paracetamol on iNOS were also seen in experiments with adult rat hepatocytes proliferating in vitro independent of the presence of LiCl (Figure 7). It is well established that peroxynitrite can nitrate paracetamol to 3-nitroparacetamol [33]. In the paracetamol-containing samples from both the recombinant heNOS and the rat hepatocytes iNOS, no 3-[^{15}N]nitroparacetamol was detected by GC-MS/MS above the limit of quantitation (about 1 nM), suggesting no formation of peroxynitrite (data not shown).

4. Discussion

4.1. General Remarks and Aim of the Study. Paracetamol is generally assumed to increase oxidative stress and is therefore

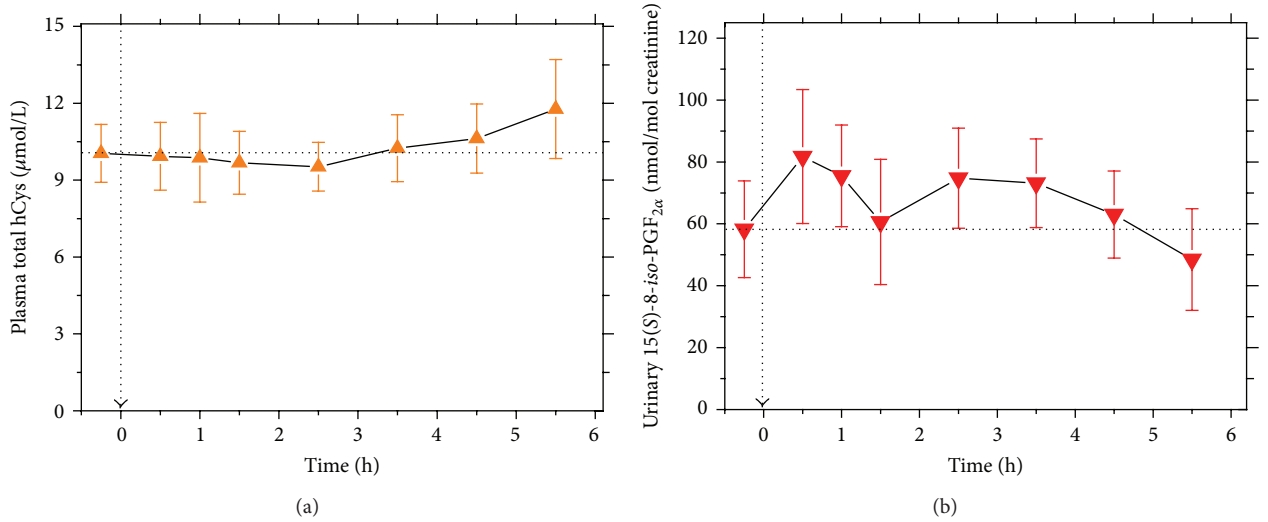

FIGURE 3: Effect of a 3 g single oral dose of paracetamol on oxidative stress in four healthy volunteers (time zero and baseline value are indicated by dashed arrows). (a) Plasma total homocysteine (hCys) concentration and (b) creatinine-corrected urinary excretion rate of 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ (15(*S*)-8-*iso*-$PGF_{2\alpha}$) as a measure of oxidative stress. Data are shown as mean ± SEM.

commonly used in animal models of oxidative stress, in which paracetamol is administered in exorbitant high doses [21]. Whether paracetamol, when administered at therapeutic doses, also acts as a prooxidant is unknown. Paracetamol is known to interact with many enzymes such as CYP, COX, and NOS, which themselves are known to contribute to oxidative stress, for instance, by producing superoxide radical anions. While the inhibitory effect of paracetamol on prostacyclin synthesis in vivo in humans is well established [16], its effects on thromboxane and NO synthesis as well as on CYP activity are incompletely understood. This may be due to insufficiently high intracellular paracetamol concentrations when this drug is administered in therapeutic doses, for instance, by oral administration of a 500 mg paracetamol tablet. The aim of the present work was to investigate in healthy humans the effects of high-dosed paracetamol (i.e., 3 g) on the activity of the COX, NOS, and CYP, as well as on oxidative stress. Given the well-known hepatotoxicity of paracetamol, only four healthy subjects were enrolled in the human study. By using paracetamol concentrations that are expected to prevail for a considerable period of time after administration of a single 3 g oral dose to humans, we investigated the effects of paracetamol at suprapharmacological concentrations on the activity of two NOS isoforms in vitro, that is, on recombinant human eNOS and iNOS in rat hepatocytes.

4.2. Effects of Paracetamol on the Cyclooxygenase Pathway. Considering a mean fraction (oral bioavailability, *F*) value of 88% for paracetamol [32], its mean distribution volume (V_D) in the volunteers of the human study described in this paper is estimated to be 88 L. This value is almost 25 times higher than the estimated volunteers' plasma volume and suggests that paracetamol may reach concentrations up to about 5000 μM in other body compartments except for red blood cells. Such high concentrations would be high enough to inhibit prostacyclin (PGI_2) and thromboxane (TxA_2) synthesis in endothelial cells and platelets, respectively [10].

Indeed, paracetamol, at the high single oral dose of 3 g, potently inhibited PGHS-catalyzed synthesis of PGI_2, a potent vasodilator and inhibitor of platelet aggregation. By contrast, the synthesis of TxA_2, a potent vasoconstrictor and platelet activator, was found not to be significantly inhibited by paracetamol in four subjects. Relative effects of drugs on PGI_2 and TxA_2 synthesis are commonly estimated by using the molar ratio of the prostanoids [34]. In our study, oral administration of a single 3 g oral paracetamol dose decreased the average PGI_2/TxA_2 molar ratio from about 0.6 before administration to values ranging between 0.4 and 0.2 after administration, thus shifting the vasodilatatory (PGI_2)/vasoconstrictory (TxA_2) balance at the cost of vasodilatation. A consequence of such a shift may be an increase of blood pressure. Indeed, Sudano and colleagues found that chronic administration of paracetamol to CAD patients at a lower dose (1 g TID for 2 weeks) than in the present study resulted in small blood pressure increase [9]. It is worth mentioning that in the study by Sudano et al. [9] considerably lower plasma paracetamol concentrations had been reached in comparison to those we measured in our study. In confirmation of previous studies [9, 15], we found that excretion of PGE_2 did not change upon paracetamol administration, suggesting that even a high dose of 3 g paracetamol did not alter significantly renal PGE_2 production in the volunteers.

4.3. Effects of Paracetamol on the L-Arginine/NO Pathway. The effects of paracetamol on NOS expression and activity have been studied by several groups. Yet, the observations are contradictory [17–20]. In our human study,

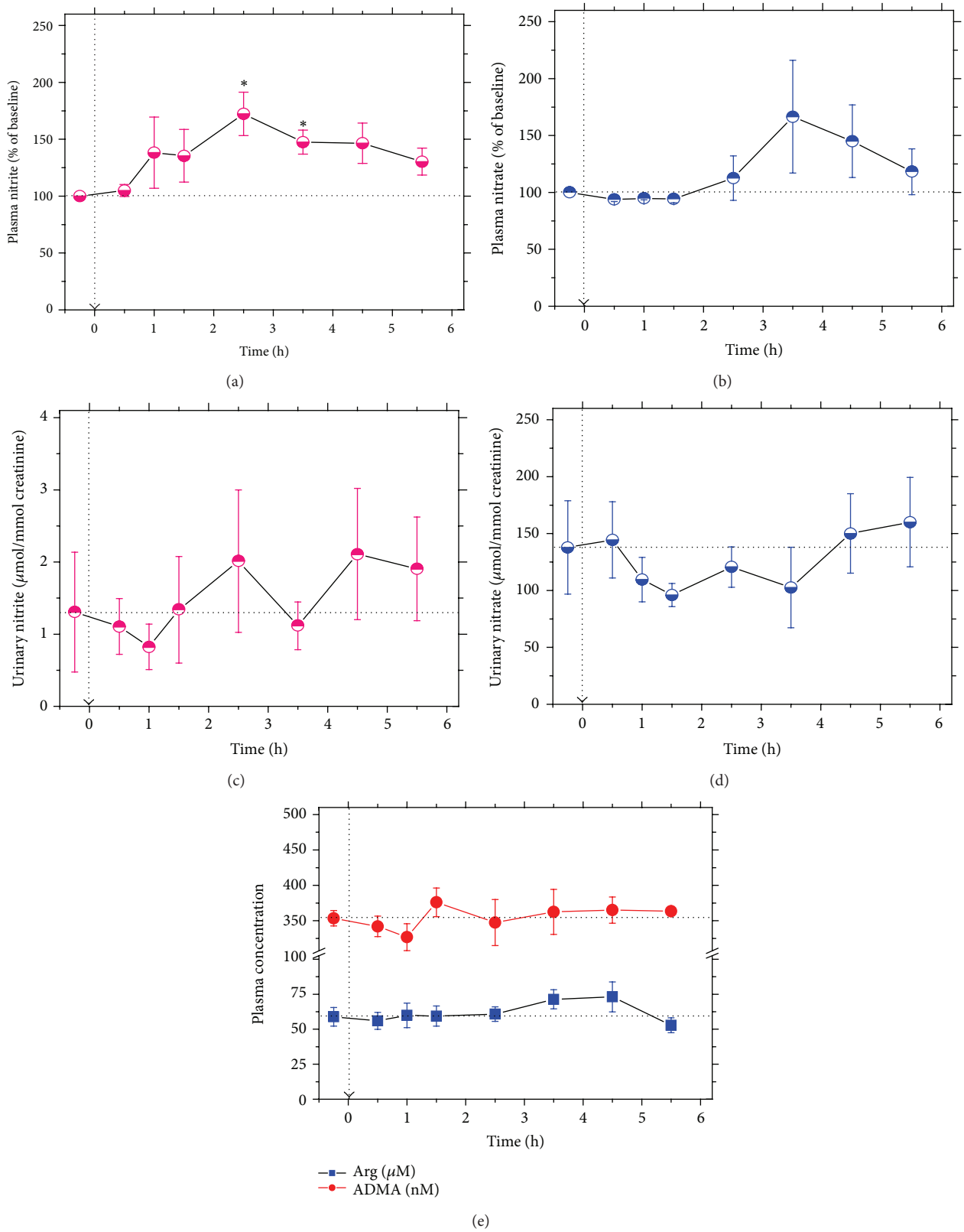

Figure 4: Effect of a 3 g single oral dose of paracetamol on plasma (a) and urine (c) nitrite, plasma (b) and urine (d) nitrate, and plasma arginine (Arg) and asymmetric dimethylarginine (ADMA) (e) in four healthy volunteers (time zero and baseline values are indicated by dashed lines). Data in plasma are shown as percentage changes of the baseline plasma nitrite concentrations (1.26, 3.04, 3.76, and 4.05 μM) and baseline plasma nitrate concentrations (37.3, 42.5, 26.8, and 52.9 μM), respectively. Data are shown as mean ± SEM. An asterisk indicates statistical significance ($P < 0.05$) compared to the 0.5 h values.

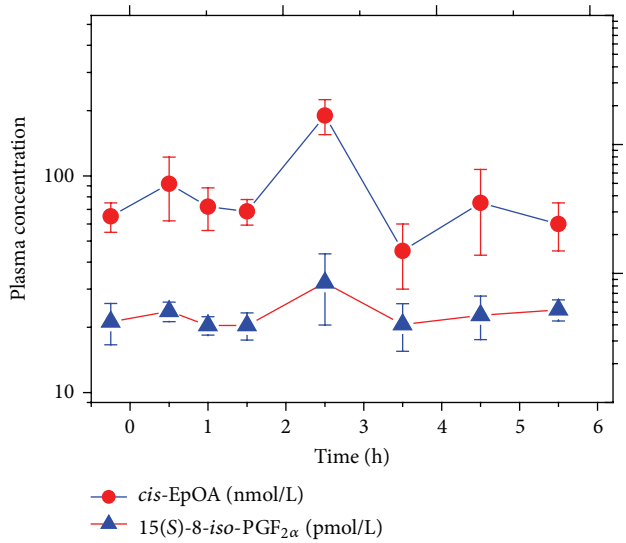

FIGURE 5: Effect of a 3 g single oral dose of paracetamol on serum *cis*-epoxyoctadecanoic acid (*cis*-EpOA) and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in four healthy volunteers (time zero and baseline value are indicated by dashed lines). Data are shown as mean ± SEM. Note the logarithmic scale on the *y*-axis. Only the 2.5 h concentration of *cis*-EpOA was statistically significantly different ($P < 0.05$) compared to the baseline.

paracetamol increased temporarily the concentration of nitrite in plasma. As the major fraction of circulating nitrite may originate from NO produced in the endothelium [23], our in vivo results may indicate that paracetamol increased eNOS activity and/or eNOS expression 2.5 to 3.5 h after administration. Yet, alternative ways such as paracetamol-induced reduction of nitrate to nitrite may have also increased plasma nitrite concentrations in the volunteers. Paracetamol did not change the plasma concentration of two other main parameters of the L-arginine/NO pathway, that is, L-arginine and ADMA. In vitro, paracetamol had only a very weak inhibitory effect on isolated recombinant heNOS and on iNOS in adult rat hepatocytes proliferating in vitro. LiCl that is known to induce expression and activity of iNOS in rat hepatocytes [31] increased iNOS activity but did not alter NO bioavailability. Thus, neither paracetamol nor LiCl influenced iNOS-related oxidative stress in rat hepatocytes.

4.4. Effects of Paracetamol on the Cytochrome P450 Pathway. Paracetamol is oxidized by the CYP family to NAPQI (*N*-acetyl-*p*-benzoquinone imine), the toxic intermediate of paracetamol. Unsaturated fatty acids including arachidonic acid and oleic acid are substrates for CYP enzymes [28, 35], and some of the arachidonic acid epoxides are vasoactive compounds [35]. At high concentrations (e.g., 1000 μM), paracetamol may inhibit the activity of CYP isoforms. In our human study, paracetamol increased temporarily the plasma concentration of the oleic acid oxide *cis*-EpOA. As *cis*-EpOA is a marker of CYP activity in humans [28], this finding may suggest that paracetamol increases CYP activity for a very short period of time. Another explanation for the very short-lasting increase in plasma *cis*-EpOA concentration could be activation of extracellular phospholipase A_2 (PLA_2) activity or release of hepatic PLA_2 into the blood stream by paracetamol, because a considerable fraction of *cis*-EpOA is found esterified to human serum lipids [28]. The latter explanation is supported by the finding that the plasma concentration of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ displayed a similar course including the sharp maximum like *cis*-EpOA in the present human study. It is worth mentioning that both 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *cis*-EpOA are released in parallel from serum lipids upon incubation with PLA_2 [28]. At present, very little is known about paracetamol effects on PLA_2 activity and/or expression. In contrast to indomethacin, paracetamol (at 1000 μM) was found not to inhibit extracellular PLA_2 activity as measured using radiolabelled oleic acid esterified to *E. coli* membranes [36]. In mice, hepatotoxicity induced by paracetamol at a dose of 400 mg/kg, that is, about 10 times higher than in our human study, was found to be associated with a time-dependent mode with increased secretion of hepatic PLA_2 which was exacerbated in the absence of hepatic COX-2 [37]. Thus, the temporary increase in *cis*-EpOA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ observed in our study may be due to paracetamol-induced short-term hepatotoxicity in the healthy subjects.

4.5. Effects of Paracetamol on Oxidative Stress. In the human study, paracetamol (3 g) did not increase oxidative stress as assessed by measuring urinary excretion of the oxidative stress biomarker 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [21, 22]. As discussed above, the sharp and short-lasting increase in the plasma concentration of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is likely to be due to temporary release of PLA_2 from the liver and/or due to activation of extracellular PLA_2. At this high dose, paracetamol did not increase plasma total hCys which is generally assumed to be associated with oxidative stress. Given the ROS-scavenging phenolic moiety of paracetamol (*N*-acetyl-*p*-aminophenol), the failure of paracetamol to enhance oxidative stress seems reasonable. The F_2-isoprostane 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is known to be produced from AA by the catalytical action of COX [38]. In contrast to acetylsalicylic acid, indomethacin, and celecoxib [25, 39], our study indicates that paracetamol (3 g) does not inhibit COX-dependent formation of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in humans.

5. Conclusion

We investigated in vitro and in vivo effects of paracetamol, an analgesic and antipyretic phenolic drug, on the L-Arg/NO, AA/COX, and CYP biochemical pathways and on oxidative stress. At the high single oral dose of 3 g, paracetamol did not alter oxidative stress in vivo. At supratherapeutic concentrations, paracetamol also did not alter oxidative stress in vitro as revealed by the unchanged nitrite-to-nitrate molar ratios measured in incubation mixtures of recombinant heNOS and in cultures of adult rat hepatocytes that express iNOS. The potent PGI_2 inhibition by high-dosed paracetamol

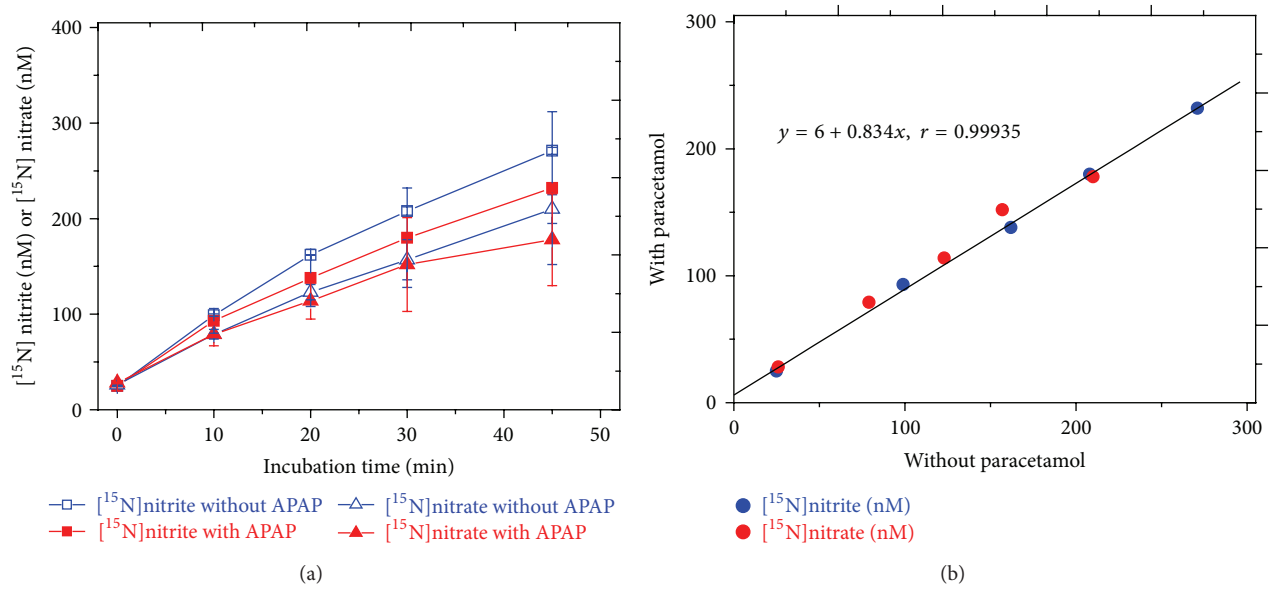

FIGURE 6: Effect of paracetamol (APAP) at 100 μM (15 mg/L) on the formation of [^{15}N]nitrite and [^{15}N]nitrate in an incubation mixture of recombinant heNOS upon incubation time (a) and linear regression analysis between [^{15}N]nitrite and [^{15}N]nitrate concentrations measured in the presence and absence of paracetamol (b). Data in (a) are shown as mean ± SD from two independent experiments; no statistical analysis was performed.

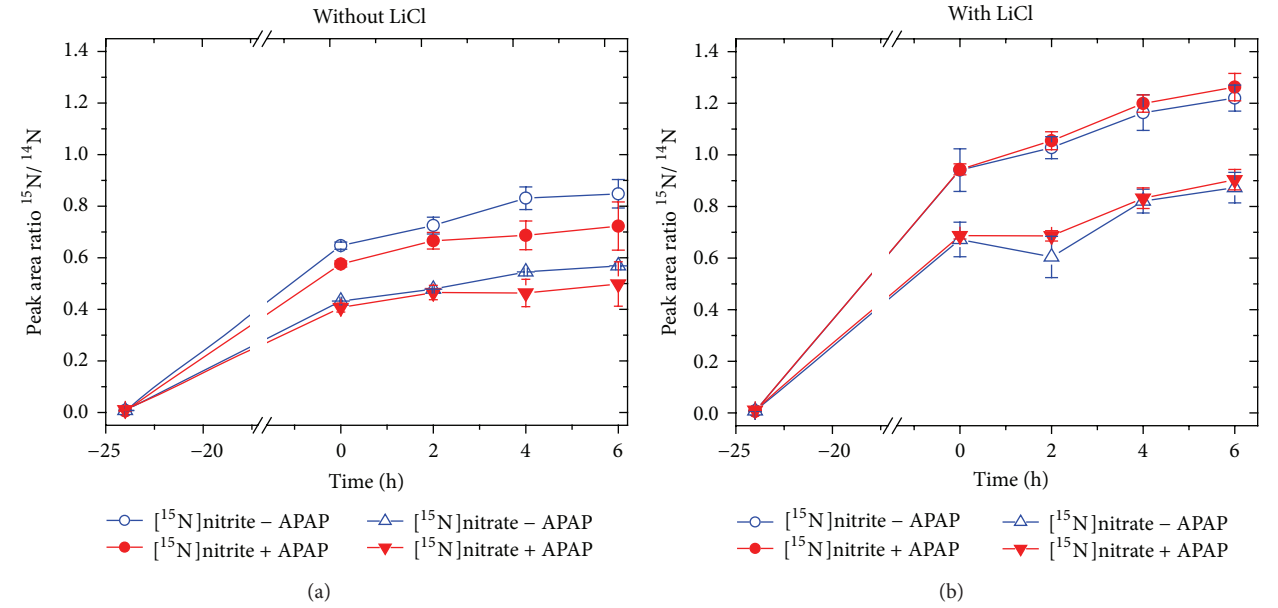

FIGURE 7: Effect of paracetamol (APAP) at 100 μM (15 mg/L) on the peak area ratio of m/z 47 for [^{15}N]nitrite to m/z 46 for [^{14}N]nitrite (a) and on the peak area ratio of m/z 63 for [^{15}N]nitrate to m/z 62 for [^{14}N]nitrate (b) upon incubation of adult rat hepatocytes with L-[*guanidine*-$^{15}N_2$]-arginine (5 mM) in the absence and in the presence of LiCl (1 mM) for the indicated times at 37°C as described elsewhere [8]. Reactions were terminated by addition of 400 μL aliquots of ice cold acetone and samples were further processed for GC-MS analysis. Data are shown as mean ± SD from three independent experiments; no statistical analysis was performed.

in the healthy subjects of the present study suggests that the relatively small increase in blood pressure seen in CAD patients by others [9] is likely to be due to compensatory mechanisms that involve enhanced formation of vasodilators. Potential candidates are NO and epoxyeicosatrienoic acids (EETs). In the circulation, NO can be produced from L-arginine by the catalytic action of eNOS and/or from nitrite/nitrate. EETs are produced from arachidonic acid by the catalytic action of the CYP family [34]. Our results suggest that in healthy subjects NO may compensate the

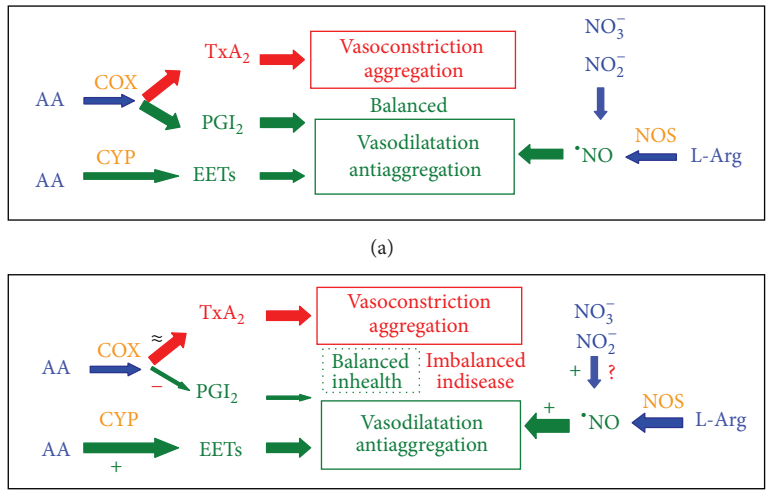

Figure 8: Simplified scheme showing the proposed contribution of three major enzymatic pathways to the vasoconstriction, vasodilatation, and aggregation in the vasculature without (a) and with (b) administration of paracetamol (acetaminophen) in health and disease. In platelets, arachidonic acid (AA) is converted by COX-1 to the vasoconstrictor and aggregator TxA_2. In endothelial cells, AA is converted by COX-2 to PGI_2, and L-Arg is oxidized by NOS to $^{\bullet}NO$; PGI_2 and $^{\bullet}NO$ are both vasodilatators and antiaggregators. AA is converted by CYP epoxygenases (CYP) to the vasodilatators epoxyeicosatrienoic acids (EETs). (a) In the absence of COX inhibitors including paracetamol and in health, production of TxA_2, PGI_2, $^{\bullet}NO$, and EETs guarantees a balance between vasoconstriction/aggregation and vasodilatation/antiaggregation. (b) Paracetamol and other COX inhibitors shift this balance in favour of COX-dependent vasoconstriction/aggregation. In response to this shift, $^{\bullet}NO$ and EETs formation is increased in order to compensate the imbalance. In health, this compensation succeeds and blood pressure and platelet aggregation do not change. In endothelium dysfunction-related diseases, such as coronary artery disease (CAD), this compensation is insufficient and leads to moderate increase in blood pressure [9]. −, +, and ≈ mean inhibition, activation, and no remarkable change, respectively. The thickness of the arrows is quantitative but not true to scale measure of the contribution of the individual pathways and paracetamol.

loss of the vasodilatory and antiaggregatory prostacyclin caused by high-dosed paracetamol (Figure 8). Also, paracetamol does not increase oxidative stress even when given at suprapharmacological doses. We assume that in healthy humans the paracetamol-induced shift of the PGI_2/TxA_2 balance is counteracted by concomitant increase in circulating NO production. The underlying mechanisms remain elusive. Possible contributing mechanisms may include elevation of NO in the endothelium and conversion of nitrate to nitrite and its consecutive reduction to NO. In patients suffering from cardiovascular diseases, that is, with dysfunctional endothelium, paracetamol only partially counteracts its unfavorable vasodilatory/vasoconstrictory effect via NO.

Abbreviations

AA: Arachidonic acid
ADMA: Asymmetric dimethylarginine
APAP: Acetaminophen (i.e., paracetamol)
Arg: Arginine
ASA: Acetylsalicylic acid
CAD: Coronary artery disease
CYP: Cytochrome P450
COX: Cyclooxygenase
EETs: Epoxyeicosatrienoic acids
FPIA: Fluorescence polarimetry immunoassay
GC-MS: Gas chromatography-mass spectrometry
GC-MS/MS: Gas chromatography-tandem mass spectrometry
HUVECs: Human umbilical vein endothelial cells
tHyc: Total homocysteine
NO: Nitric oxide
NOS: Nitric oxide synthase
eNOS: Endothelial nitric oxide synthase
heNOS: Human endothelial nitric oxide synthase
iNOS: Inducible nitric oxide synthase
nNOS: Neuronal nitric oxide synthase
NSAIDs: Nonsteroidal anti-inflammatory drugs
PG: Prostaglandin
PGE_2: Prostaglandin E_2
PGE-MUM: Prostaglandin E-major urinary metabolite
PGHS: Prostaglandin H synthase
PGI_2: Prostacyclin
PLA_2: Phospholipase A_2
QC: Quality control
ROS: Reactive oxygen species
SD: Standard deviation
SEM: Standard error of the mean
TID: ter in di (three times a day)
TxA_2: Thromboxane A_2.

Conflict of Interests

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

Authors' Contribution

Dimitrios Tsikas conceived and designed all experiments described in the paper. Arne Trettin and Anke Böhmer performed in vitro experiments on recombinant enzymes and hepatocytes, carried out biochemical analyses, and wrote parts of the paper. Maria-Theresia Suchy performed biochemical analyses of the samples obtained in the human study. Irmelin Probst managed and supervised the experiments on hepatocytes. All authors read and approved the final version of the paper.

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Clinical Study

L-Arginine/NO Pathway Is Altered in Children with Haemolytic-Uraemic Syndrome (HUS)

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The haemolytic uraemic syndrome (HUS) is the most frequent cause of acute renal failure in childhood. We investigated L-arginine/NO pathway in 12 children with typical HUS and 12 age-matched healthy control subjects. Nitrite and nitrate, the major NO metabolites in plasma and urine, asymmetric dimethylarginine (ADMA) in plasma and urine, and dimethylamine (DMA) in urine were determined by GC-MS and GC-MS/MS techniques. Urinary measurements were corrected for creatinine excretion. Plasma nitrate was significantly higher in HUS patients compared to healthy controls ($P = 0.021$), whereas urine nitrate was borderline lower in HUS patients compared to healthy controls ($P = 0.24$). ADMA plasma concentrations were insignificantly lower, but urine ADMA levels were significantly lower in the HUS patients ($P = 0.019$). Urinary DMA was not significantly elevated. In HUS patients, nitrate ($R = 0.91$) but not nitrite, L-arginine, or ADMA concentrations in plasma correlated with free haemoglobin concentration. Our results suggest that both NO production and ADMA synthesis are decreased in children with typical HUS. We hypothesize that in the circulation of children with HUS a vicious circle between the L-arginine/NO pathway and free haemoglobin-mediated oxidative stress exists. Disruption of this vicious circle by drugs that release NO and/or sulphydryl groups-containing drugs may offer new therapeutic options in HUS.

1. Introduction

Haemolytic-uraemic syndrome (HUS) is the most frequent cause of acute renal failure in childhood [1]. HUS is defined by the triad of haemolytic anaemia, acute renal failure, and thrombocytopenia. HUS in childhood is predominantly induced by an infection with verocytotoxin- (Shiga-like toxin-) producing bacteria, typically *Escherichia coli*. HUS primarily occurs in children one to 10 years of age with an average annual incidence of one to three cases per 100,000 children and a survival rate of nearly 95% [2]. Beside the outbreak of Shiga-toxin producing *E. coli* O104:H4 in 2011 [3], in Austria and Germany, the incidence is 0.4 : 100,000 and 0.7 : 100,000, respectively [4]. The disease begins after an incubation period of 4 to 7 days with abrupt onset of bloody diarrhoea and abdominal pain. Two to ten days later, microangiopathy, haemolytic anaemia, thrombocytopenia, and acute renal failure develop. Specific pathological findings in HUS patients with acute renal failure are glomerular microthrombi [5, 6]. HUS microangiopathy can involve almost any organ, but damage to kidneys and central nervous system cause the most severe clinical problems [7]. Despite

the often dramatic clinical presentation, the overall outcome of childhood HUS is relatively good. Mortality is less than 5%, and 75% of patients show complete remissions [8]. Relapses are rare.

Shiga-like toxin binds to the glycosphingolipid globotriaosylceramide [9], thus interfering with protein synthesis in endothelial cells. Disordered von Willebrand factor, platelet activation via platelet-activating factor, interleukins, and nitric oxide (NO) may also contribute to the disorder [7]. NO has multiple functions including regulation of vascular tone, neurotransmission, and inhibition of platelet aggregation and leukocyte adhesion [10]. Antiproliferative and antiatherosclerotic effects have also been ascribed to NO [11].

NO is a short-lived free radical which is produced in all types of cells including endothelial cells. In vivo, NO is rapidly oxidized to nitrate and nitrite, which circulate in blood and are excreted in the urine [12]. Under certain conditions circulating nitrite and nitrate and excretory nitrate are suitable indicators and measures of NO synthesis [13]. Thus, nitrate in urine is considered a useful measure of whole body NO synthesis, whereas circulating nitrite rather reflects endothelium-dependent NO synthesis. NO is generated from the amino acid L-arginine by NO synthases (NOS) isoforms, including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). These enzymes oxidize the imino group of the terminal guanidine group of L-arginine to NO, with L-citrulline being the second reaction product [14].

NOS activity is regulated by endogenous inhibitors, with asymmetric dimethylarginine (ADMA) being the most important [15]. ADMA is generated by methylation of protein-associated L-arginine catalyzed by N-methyl protein transferases followed by regular proteolysis [16]. Unchanged ADMA is excreted by the kidneys, but the greatest part of endogenously produced ADMA (about 90%) is excreted in the urine as dimethylamine (DMA) after hydrolysis by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [15], predominantly in kidney and liver. High circulating ADMA levels were found in many diseases including chronic renal failure [17].

In adult patients suffering from different microangiopathic diseases NO synthesis seems to be increased [18]. Yet, in childhood data on the L-arginine/NO pathway is rare. We recently reported reference data for the different members of the NO family in childhood [19]. We also studied these parameters in children suffering from renal diseases [20]. As microangiopathy also occurs in HUS patients, we were interested to see if the L-arginine/NO pathway is altered in children with HUS. It is worth mentioning that in the acute phase of HUS, erythrocytes are exposed to elevated oxidative stress that could contribute to haemolysis directly through oxidative damage and/or decreasing membrane fluidity [21]. More recently, it was demonstrated that during the acute phase of HUS in humans oxidative stress is elevated when measured as lipid peroxidation products in plasma [22]. In mice, Shiga toxin 2 was shown to increase oxidative stress and antioxidants such as *N*-acetylcysteine were found to ameliorate Shiga toxin-induced oxidative stress measured as malondialdehyde and to decrease renal damage [23]. In a patient, a 9-year-old girl, suffering from HUS after transplantation transdermal application of isosorbide dinitrate (ISDN), a NO-releasing drug was found to be very effective in ameliorating haemolysis and in increasing the number of platelets [24]. This interesting finding indicates that NO may play a protective role in HUS. In consideration of the potential involvement of the L-arginine/NO pathway in children suffering from typical HUS, we wanted to characterize quantitatively and comprehensibly the status of HUS by measuring several members of the L-arginine/NO pathway in plasma and urine. Healthy children served as the control group in the present study. As nitrite in human urine may potentially indicate nitrative stress [25], we quantitated urinary nitrite in addition to urinary nitrate which is a measure of whole-body NO synthesis [13].

2. Materials and Methods

Twelve children with typical HUS (5 girls, 7 boys; mean age 3.6 ± 3.5 years) and twelve age-matched healthy controls were included. The clinical characteristics of the patients are summarized in Table 1. Patients were treated symptomatically with diuretics and dialysis. If arterial hypertension was present they received antihypertensive drugs (Table 1). Patients were discharged about two weeks after the end of dialysis which was performed for 5 to 30 days (Table 1). Blood and, where possible, urine were taken at the first or second day of inpatient stay. The study was approved by the Ethics Committee of the Hannover Medical School and written consent was obtained from the parents.

ADMA in plasma and urine and L-arginine in plasma were determined by GC-MS/MS and GC-MS, respectively, as described elsewhere [26]. Nitrite and nitrate in plasma and urine were determined simultaneously by GC-MS as described previously [27]. Urinary creatinine was determined by GC-MS [28]. DMA in urine was determined by GC-MS as described recently [29]. Urinary excretion of the analytes was corrected for creatinine excretion and data are presented as μmol of the analyte per mmol of creatinine.

As a measure of endothelial damage, circulating endothelial cells were detected in blood at the time of admission as described by us elsewhere [30].

Data from patients and healthy controls were compared using the Mann-Whitney test (SPSS, version 16). Data are presented as mean ± SD. Values of $P < 0.05$ were considered significant.

3. Results

Upon admission, the number of circulating endothelial cells in the HUS childrens' blood was 44 per mL (median, range 0 to 800 per mL), indicating endothelial damage [30]. At time of discharge, the number of circulating endothelial cells decreased to 24 per mL (median, range 0 to 180 per mL).

In children with HUS, plasma nitrate (61.9 ± 18.9 versus 41.5 ± 13.1 μM, $P = 0.021$) and plasma nitrite (3.1 ± 0.8 versus 2.4 ± 1.1 μM, $P = 0.017$) were significantly increased, as compared to the healthy controls (Figure 1). Excretion rates of nitrate were insignificantly lower in the HUS patients as

Table 1: Clinical characteristics of the haemolytic uraemic syndrome (HUS) patients on the 1st or 2nd day of admission and duration of peritoneal dialysis.

Patient number	Duration of peritoneal dialysis (days)	Neurological events	Hypertension	Serum creatinine (μM)	Serum urea (mM)	Urine albumin (g/L)	Total hemoglobin (g/dL)	Free hemoglobin (mg/L)	Lactate dehydrogenase (U/L)
1	0	No	No	108	15.4	3.7	6.6	429	2448
2	7	No	No	394	28.4	0.37	7.6	196	968
3	30	Ataxy	Yes	256	35.0	0.05	6.8	127	3789
4	5	No	No	458	41.9	0.41	5.9	165	2606
5	9	EEG alteration	No	132	20.4	Not measured	6.8	155	1462
6	24	No	Yes	209	21.0	0.89	5.7	Not measured	2611
7	30	Status epilepticus	No	304	26.3	0.24	4.8	Not measured	2593
8	10	No	No	50	7.8	4.45	6.9	84	3090
9	8	EEG alteration	No	528	39.4	Not measured	13.0	Not measured	2003
10	6	No	No	600	68.0	Not measured	8.2	Not measured	Not measured
11	20	No	Yes	373	19.8	3.4	6.0	Not measured	2662
12	14	EEG alteration	No	296	28.4	4.97	6.2	284	2379

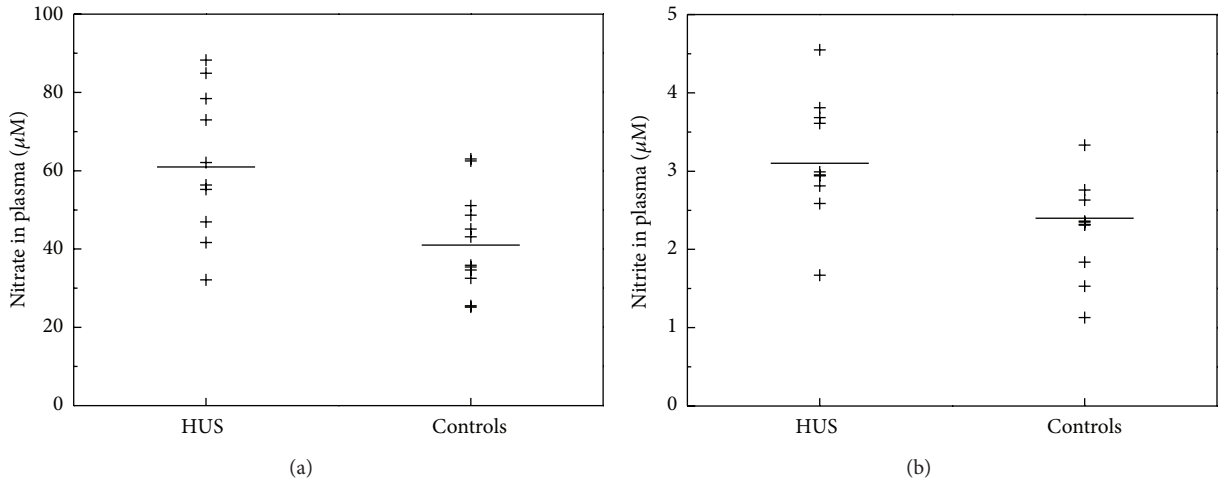

Figure 1: Plasma concentrations of nitrate (a) and nitrite (b) in children with haemolytic-uraemic syndrome (HUS, $n = 12$) and in healthy children (controls, $m = 12$). Horizontal bars indicate the mean values. Nitrate ($P = 0.021$) and nitrite concentrations were significantly ($P = 0.017$) higher in HUS as compared with controls.

compared to the healthy controls (100 ± 27 versus 187 ± 207 μmol/mmol, $P = 0.24$) (Figure 2(a)). Urinary nitrite did not differ between HUS and healthy controls (0.3 ± 0.2 versus 0.2 ± 0.2 μmol/mmol, $P = 0.58$) (Figure 2(b)).

Plasma ADMA concentrations were insignificantly lower in the HUS children as compared to the healthy children (666 ± 160 versus 746 ± 208 nM, $P = 0.32$) (Figure 3(a)). However, renal excretion rate of ADMA was significantly lower in patients with HUS compared to healthy controls (3.3 ± 2.5, $n = 5$, versus 10.1 ± 6.5, $n = 9$) μmol/mmol, $P = 0.019$) (Figure 3(b)). Excretion rate of DMA in urine was insignificantly higher in patients with HUS, that is, 13.7 ± 14.4 ($n = 4$) in comparison to 8.4 ± 5.6 ($n = 5$) μmol/mmol in healthy children ($P = 1.0$) (Figure 4). Plasma L-arginine plasma levels were not statistically different between the two groups (Figure 5; $P = 0.48$).

In the HUS patients, there was a close positive correlation ($R = 0.91$, $P = 0.01$) between plasma nitrate concentration (y) and plasma free haemoglobin concentration (x) with the regression equation $y = 25 + 0.15x$; by contrast, plasma

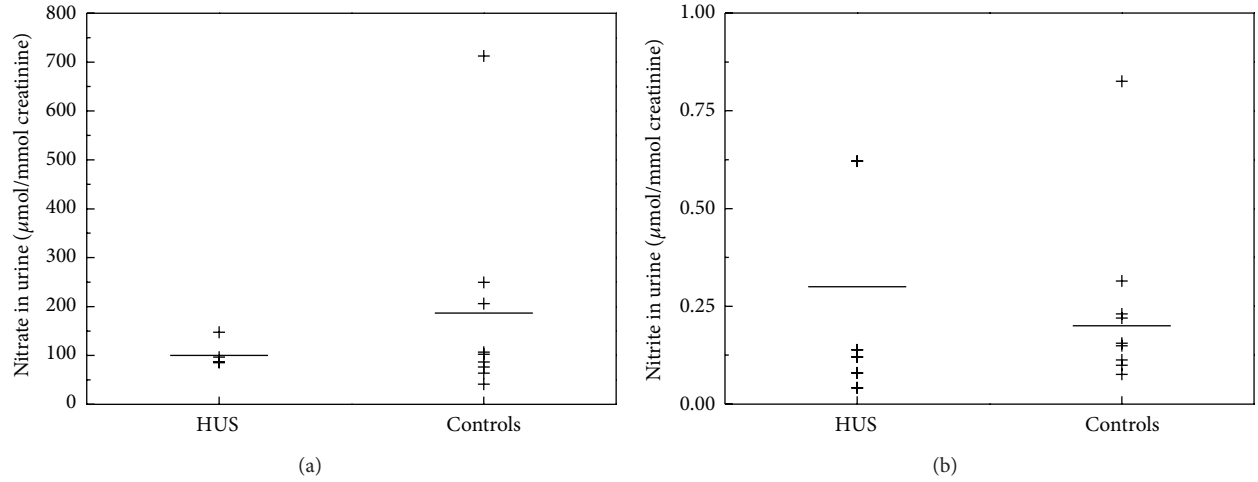

Figure 2: Urinary excretion of nitrate (a) and nitrite (b) in children with haemolytic-uraemic syndrome (HUS, n) and in healthy children (controls, m). Horizontal bars indicate the mean values. Nitrate and nitrite excretion rates did not differ significantly between HUS and control ($P = 0.24$ and $P = 0.58$, resp.; each $n = 5$, $m = 9$).

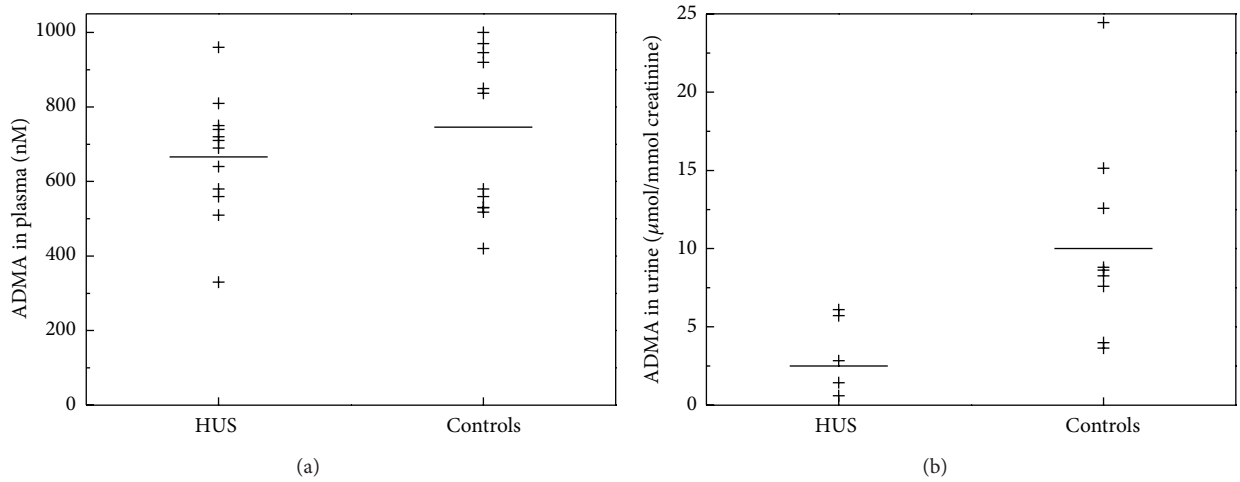

Figure 3: Plasma concentrations (a) and urine excretion rates (b) of ADMA in children with haemolytic-uraemic syndrome (HUS, n) and in healthy children (controls, m). Horizontal bars indicate the mean values. Plasma ADMA concentrations were insignificantly lower in HUS as compared with controls ($P = 0.32$; $n = 12$, $m = 12$). Urinary excretion of ADMA was significantly lower in HUS as compared with controls ($P = 0.019$; $n = 5$, $m = 9$).

nitrite, L-arginine, and ADMA concentration did not correlate with plasma free haemoglobin concentration (Figure 6).

4. Discussion

The L-arginine/NO pathway plays an important role in renal failure, infection, and microangiopathy [18, 31, 32]. In adult patients with microangiopathy endogenous NO production seems to be elevated [18]. In the present study we investigated the status of the L-arginine/NO pathway in children with typical haemolytic uraemic syndrome (HUS), which is associated with haemolytic anaemia, acute renal failure, and thrombocytopenia.

The number of free circulating endothelial cells in children with HUS at the time of admission was much higher than that commonly found in healthy individuals [30]. These expected findings suggest that endothelial damage occurred in the HUS patients investigated in the present study. At time of discharge, the number of circulating endothelial cells decreased to normal levels, indicating improvement of endothelial dysfunction.

To study the L-arginine/NO pathway we obtained blood and urine samples at the first or second day of inpatient stay. In the plasma samples of our patients with typical HUS we observed significantly increased concentrations of nitrate and nitrite compared to healthy children. In former studies of our groups, nitrate and nitrite concentrations were not elevated in children with syndromic [33] and nonsyndromic focal-segmental-glomerulosclerosis (FSGS) or in children with non-FSGS renal diseases [20]. Taken all together, impaired renal function in HUS patients is likely to be a reason for accumulating plasma nitrite and nitrate concentrations

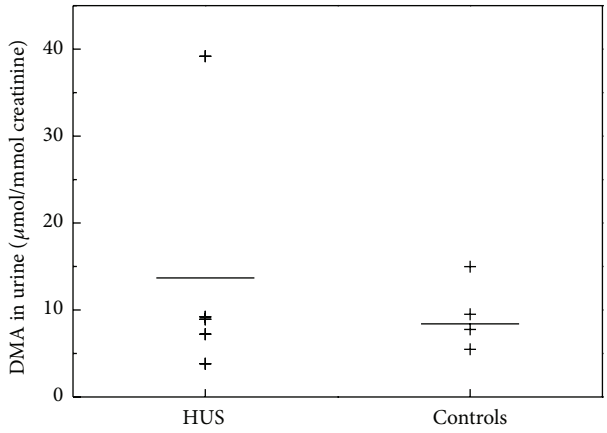

Figure 4: Urinary excretion rate of DMA in children with haemolytic-uraemic syndrome (HUS; $n = 4$) and in healthy children (controls, $m = 5$). Horizontal bars indicate the mean values. DMA levels were insignificantly higher in HUS as compared with controls ($P = 1.0$).

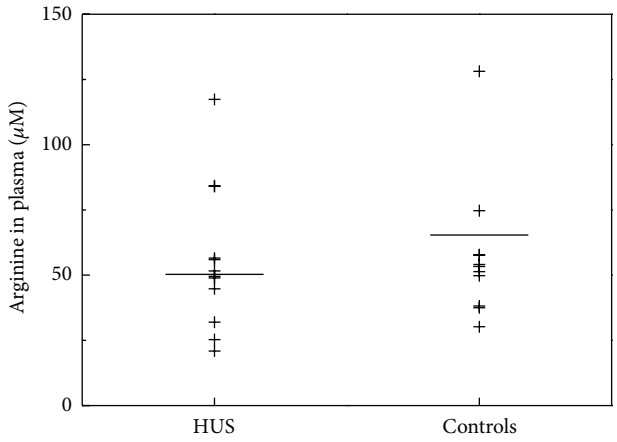

Figure 5: Plasma concentrations of arginine in children with haemolytic-uraemic syndrome (HUS; n) and in healthy age-matched children (controls, m). Horizontal bars indicate the mean values. Arginine concentrations did not differ between patients and healthy controls ($P = 0.48$; $n = 12$, $m = 12$).

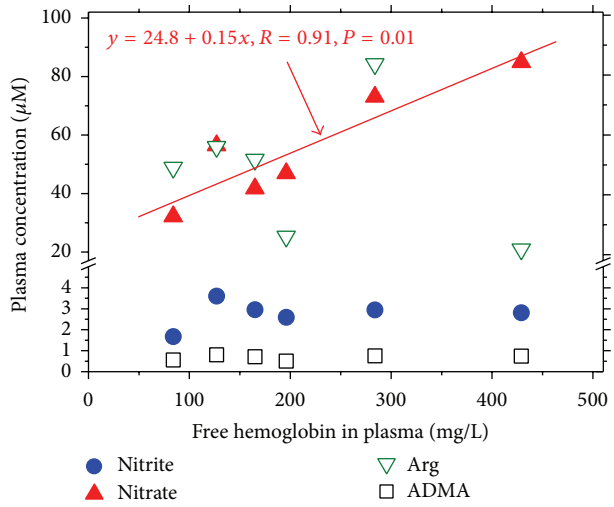

Figure 6: Relationship between nitrite, nitrate, L-arginine, or ADMA in plasma and free hemoglobin in plasma of the children with haemolytic-uraemic syndrome investigated in the present study.

rather than an enhanced NO synthesis (Figure 7). In the children of the present study, we did not measure cGMP, the second messenger of NO, in plasma or urine samples. In forthcoming studies, measurement of cGMP in plasma of HUS children could provide additional, valuable information about NO biosynthesis/bioavailability in this syndrome, although circulating or urinary cGMP and NO synthesis or bioactivity are not dependable correlates.

Unfortunately, urine samples were not available from all HUS children and the power of the biochemical parameters measured in urine samples is considered rather low. While plasma ADMA concentrations were not increased in the HUS patients, urinary excretion rate of ADMA in HUS patients was almost threefold lower than in healthy children. Analogous to urinary nitrate, excretion of unchanged ADMA in the urine seems to be impaired most likely due to renal failure. These results and the observation that DMA excretion rates were similar in the HUS patients and the healthy controls suggest that ADMA synthesis rate is decreased in typical HUS, presumably due to impaired activity of N-methyl protein transferases and/or impaired activity of proteolytic enzymes in this syndrome (Figure 7). On the other hand, our results suggest that DDAH activity is not altered in HUS. Deficiency of L-arginine, the common precursor of NO and ADMA, is unlikely to explain the reduction in ADMA synthesis in the HUS patients, as plasma L-arginine concentrations were similar in patients and in control subjects.

Elevated NO production has been observed in a mouse model of Shiga toxin 2-induced HUS [34]. Shao et al. [35] suggested eNOS upregulation in a rat model for thrombotic microangiopathy and that elevated endothelial NO synthesis could be an important protective mechanism in thrombotic microangiopathy [35]. NO production by neutrophil leucocytes following stimulation with Shiga toxin has been found to be age-dependent with lower production in infants; the authors speculate that this may be related to the higher incidence in infants [36]. As nitrite and nitrate in plasma and/or in urine do not reflect the activity of a particular NOS isoform or organ, our results do not allow the drawing of any conclusions regarding eNOS contribution to NO in the HUS patients of healthy children. Therefore, in our HUS patients we cannot exclude elevation in expression and activity of iNOS which generates NO for purpose of antimicrobial defense, but we consider it rather very moderate.

The intact erythrocyte plays an important role both in NO inactivation through oxyhaemoglobin and in storage and transport of NO bioactivity within the circulation [37–42]. Therefore, it is likely that haemoglobin species inside as well as outside of the erythrocyte, that is, free haemoglobin, are of particular importance for NO-related biological actions

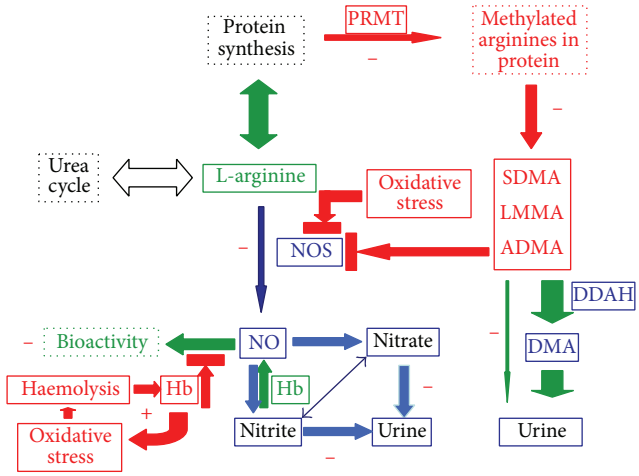

Figure 7: Proposal of the status of the L-arginine/NO pathway in the haemolytic-uraemic syndrome (HUS) in childhood. The enzyme NOS converts L-arginine into L-citrulline (not shown) and NO which plays multiple physiological roles. NO autooxidizes to nitrite. In red blood cells, NO is oxidized to nitrate. Nitrite and nitrate are excreted in the urine. By far the major part of L-arginine participates in the urea cycle and is utilized in protein synthesis. PRMT methylate L-arginine in proteins and methylated proteins are hydrolyzed to the soluble L-arginine derivates SDMA, LMMA, and ADMA. LMMA and ADMA act as inhibitors of NOS, thus controlling NO synthesis. ADMA is hydrolyzed by DDAH to L-citrulline (not shown) and DMA which is excreted in the urine. The status of the L-arginine/NO pathway can be described satisfactorily by measuring nitrite, nitrate, ADMA, L-arginine, and DMA in plasma or serum and urine. In HUS, NO and ADMA biosynthesis is diminished, whereas DDAH activity is not altered. Oxidative stress is elevated, haemolysis is increased, which releases Hb that in turn elevates oxidative stress, thus finally establishing a vicious circle. Minus means diminished; plus means increased. Abbreviations used in this Figure: ADMA: asymmetric dimethylarginine; DDAH: dimethylarginine dimethylaminohydrolase; DMA: dimethylamine; LMMA: monomethyl arginine; NOS: nitric oxide synthase; PRMT: protein arginine methyl transferase; SDMA: symmetric dimethylarginine.

in HUS patients, in a way independent of the endothelial function. Free haemoglobin in plasma may act both as a trap for NO and as a producer of NO from inorganic nitrite, the autoxidation product of NO (Figure 7). Hypoxia is not atypical for HUS. Disturbed microcirculation in HUS caused by thrombotic microangiopathy may lead to hypoxia which facilitates nitrite reduction to NO by free haemoglobin. In this situation, additionally produced NO from nitrite may be meaningful and beneficial by acting as inhibitor of aggregation as well as a vasodilatator. In addition, haemoglobin may produce vasoactive substances such as prostaglandins and thromboxane, but it may also induce oxidative stress [43]. In the present study, we found a positive correlation between nitrate and free haemoglobin in plasma. This observation together with the almost uniform distribution of nitrite and nitrate in blood cells including erythrocytes [44, 45] may argue for oxidation of NO to nitrate by free haemoglobin outside of the erythrocyte rather than for nitrate release by damaged erythrocytes. It is worthy of mention that haemolysis in our HUS patients was not higher than about 0.2%. However, because free haemoglobin in the plasma is several times more reactive against NO than haemoglobin inside of the erythrocytes, it is likely that a large fraction of NO has been oxidized to nitrate by free haemoglobin in the plasma of our HUS patients. In HUS, like in sickle cell anemia [41], haemolysis seems to be associated with harmful rather than with beneficial effects. Cell-free haemoglobin released by haemolysis may oxidize NO to biologically inactive nitrate as mentioned above. In addition, free haemoglobin may also inactivate NO by enhancing oxidative stress, for instance, by producing reactive oxygen species (ROS) such as superoxide radical anions and hydrogen peroxide. Such ROS are highly reactive and may therefore oxidize NO to peroxynitrite. This is a strong oxidant on its own and will finally decompose to nitrite, nitrate and dioxygen. It is likely that these deleterious effects of haemoglobin are not exerted in a stoichiometric manner [43].

Based on our results suggesting a decreased synthesis rate of endogenous NO in HUS, one may speculate that basal NO synthesis is insufficient to ameliorate thrombotic microangiopathy in this syndrome and that exogenous NO in pharmacological doses may therefore be required. In a 9-year-old girl suffering from HUS following bone marrow transplantation at the age of 8 months, 20 mg/day transdermal ISDN for 9 weeks ameliorated haemolysis while increasing platelet counts. The girl did not experience side effects or the disease did not recur after cessation of ISDN treatment [24]. Interestingly, many of the clinical characteristics of this girl were comparable to those of our HUS patients. Unfortunately, no data had been reported about the L-arginine/NO pathway in this girl. It is worth mentioning that ISDN was found not to increase oxidative stress in healthy young subjects when applied at a therapeutically relevant dose of 30 mg thrice a day [46]. In contrast, at this dose ISDN appeared to decrease basal nitrative stress one day after administration when measured both as soluble 3-nitro-tyrosine and 3-nitro-tyrosine-albumin, though not statistically significant [46], which are potential biomarkers for NO-dependent oxidative stress in humans [47]. Due to the limited availability of urine samples and the artefactual contribution of haemolysis to biomarkers of oxidative stress such as malondialdehyde (MDA) and the F_2-isoprostane 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [43], but not to ADMA and other members of the L-arginine/NO pathway [48], we abandoned the analysis of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the plasma and urine samples of our study.

In adults CAD patients we found that low urinary ADMA excretion rates are associated with impaired cardiac function and predict cardiovascular as well as all-cause mortality [49]. In the present study we measured in HUS children for the first time lower ADMA excretion rates than in healthy children. The potential clinical value of urinary ADMA for the diagnosis of CAD or cardiac dysfunction in adults and for the diagnosis of HUS in childhood warrants further studies.

In summary, the L-arginine/NO pathway is altered in childhood HUS. NO synthesis seems to be diminished despite a decreased synthesis of ADMA, an endogenous inhibitor of NO synthesis from L-arginine, compared to

healthy age-matched children. Free haemoglobin is likely to play an important role in the metabolism and biological activity of NO, including endothelium-derived NO, and to induce oxidative stress, for instance, lipid peroxidation in blood, thus damaging the erythrocyte membrane and causing haemolysis. Further studies are required to delineate the relative contribution of damaged erythrocytes and damaged endothelium to HUS. Application of drugs with the potential to release NO, such as the organic nitrate ISDN, could be effective as a therapeutic means in the treatment of HUS. The underlying mechanism leading to impaired NO synthesis in HUS is unclear and demands further elucidation. The potential beneficial effect of organic nitrates on haemolysis and platelets and the underlying mechanism(s) warrant further investigations. It appears that oxidative stress is elevated in HUS while NO synthesis is decreased. Supplementation of children suffering from HUS with NO-releasing drugs or drugs that increase NO synthesis and/or bioavailability and that decrease oxidative stress, such as *N*-acetylcysteine (NAC) or its more lipophilic and cell membrane-permeable *N*-acetylcysteine ethyl ester (NACET) [50], may be useful measures among others in the treatment of HUS.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contributions

Thomas Lücke designed the study. Nele Kirsten Kanzelmeyer investigated blood and urine samples and wrote the paper. Hans Hartmann evaluated neurological outcome of patients and participated in writing the paper. Lars Pape and Anne-Jule Fuchs helped to evaluate patients. Kristine Chobanyan-Jürgens investigated DMA in urine. Dimitrios Tsikas established methods to measure members of the L-arginine/NO pathway. Bernhard Vaske did the statistics. Anibh Martin Das and Jens Jordan reviewed the paper. Marion Haubitz measured circulating endothelial cells.

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Review Article

Cardiovascular Complications of Sleep Apnea: Role of Oxidative Stress

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Obstructive sleep apnea (OSA) occurs in 2% of middle-aged women and 4% of middle-aged men with a higher prevalence among obese subjects. This condition is considered as an independent risk factor for cerebrovascular and cardiovascular diseases. One of the major pathophysiological characteristics of OSA is intermittent hypoxia. Hypoxia can lead to oxidative stress and overproduction of reactive oxygen species, which can lead to endothelial dysfunction, a hallmark of atherosclerosis. Many animal models, such as the rodent model of intermittent hypoxia, mimic obstructive sleep apnea in human patients and allow more in-depth investigation of biological and cellular mechanisms of this condition. This review discusses the role of oxidative stress in cardiovascular disease resulting from OSA in humans and animal models.

1. Introduction

Obstructive sleep apnea (OSA) is a sleep-breathing disorder characterized by intermittent episodes of either complete breathing cessation for periods of ten seconds or more (apnea) or significant reductions in breathing amplitude (hypopnea). Patients are categorized as having mild, moderate, or severe OSA depending on the apnea/hypopnea index (AHI), which is the total number of apnea/hypopnea episodes per hour of sleep. In normal individuals the index is usually 5 or lower, 5–15 in mild, 15–30 in moderate, and 30 or more in severe OSA patients [1]. The severity of OSA is accompanied by significant episodes of hypoxemia and hypercapnia, where in mild patients the oxyhemoglobin saturation drops to 95% and can drop below 80% in severe cases. Risk factors for sleep apnea include obesity, craniofacial abnormalities, smoking, male gender, short neck, and menopause in women. Obesity is one of the main risk factors of sleep apnea since 60% to 90% of OSA patients are obese and there is a strong positive correlation between BMI and OSA [2]. The overlap of obesity and OSA makes the identification of OSA versus obesity contributions to cardiovascular risk more challenging. Increased adiposity and short neck add weight to the soft tissue volume within the upper airway and the neck, further increasing airway collapsibility [3].

At a population level, at least 4% males and 2% females are diagnosed with sleep apnea and its symptoms, and it is estimated that 1 of every 5 adults have OSA and that 1 of every 15 adults have moderate OSA. In females, the prevalence increases from 3% for the third decade of life to 36% in the seventh decade. In men the prevalence for the third decade is 4% and increases to 50% during the seventh decade [15]. Unfortunately, most of those who are affected by OSA remain undiagnosed despite medical advances. Polysomnography is the main tool for diagnosing patients with OSA where sleep stages are monitored along with arterial blood gases, breathing, and electrocardiogram.

The results of many clinical studies strongly suggest that OSA is an independent risk factor for cardiovascular diseases such as hypertension, coronary artery disease, stroke, and heart failure. Several mechanisms have been suggested to link OSA and vascular diseases; evidence for this includes increases in sympathetic activation, oxidative stress, inflammation, endothelial dysfunction, coagulation, and metabolic dysregulation [16]. This review describes the role of free

Table 1: Cohort studies regarding OSA and incidence of cardiovascular disease.

Cardiovascular disease	Cohort	Sample size	Duration (years)	Findings	Reference
Hypertension	WSC	893	4 or 8	Adjusted OR of AHI ≥ 15, compared with AHI = 0 : 2.89	[4]
Coronary artery disease	SHHS	4422	8.7	Significant association only on adjusted subgroup analysis of men ≤ 70. Adjusted HR for AHI ≥ 30 compared with AHI < 5 : 1.68	[5]
Stroke	WSC	1475	4 or 8	Age and sex adjusted OR for AHI ≥ 20, compared with AHI < 5 : 4.48, nonsignificant only when adjusted to BMI.	[6]
Atrial fibrillation	Sleep-clinic patients	3542	4.7	Unadjusted HR of AHI ≥ 5, compared with AHI < 5 : 2.18	[7]

AHI: apnea-hypopnea index, BMI: body mass index, HR: hazard ratio, OR: odds ratio, SHHS: Sleep Heart Health Study, WSC: Wisconsin Sleep Cohort.

radicals and oxidative stress in cardiovascular disease in OSA patients and animal models of sleep apnea.

2. Cardiovascular Consequences of Sleep Apnea

Many of the reports correlating OSA to vascular disease come from small longitudinal studies of incidental cardiovascular disease and studies evaluating the effect of CPAP intervention. However, largely due to the cost of OSA diagnosis in large population samples, many studies can only indirectly implicate OSA in the etiology of cardiovascular disease. In addition, comorbidities such as obesity and hypertension that coexist with the majority of OSA patients make the independent risk of OSA on vascular disease more difficult to assess. Table 1 summarizes some cohort studies relating OSA and incidence of cardiovascular disease.

2.1. Hypertension. Close to 35% of OSA patients have hypertension while 30% of hypertensive patients who have OSA are undiagnosed [17, 18]. In the Wisconsin Sleep Cohort, subjects with an AHI of 15 or higher had an almost 3-fold increased risk of developing hypertension when compared to control after 4 years [4]. On the other hand, the Sleep Heart Health Study (SHHS) reported that the unadjusted risk of hypertension increased with the severity of OSA after a 5-year followup, but this association was not significant after adjustment for body mass index [19]. This discrepancy in findings could be related to differences in the study sample characteristics and the techniques used to diagnose OSA. However, a recent study by Pedrosa et al. shows that OSA is the most common condition associated with resistant hypertension (64%), followed by primary aldosteronism (5.6%) and renal artery stenosis (2.4%) in 125 patients with resistant hypertension [20]. OSA is now included as one of the main causes of hypertension in the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure [21]. Nasal continuous positive airway pressure (nCPAP) treatment reduces mean arterial blood pressure by 10 mm Hg in OSA patients both at night and day, a benefit that was lost when nCPAP treatment was subtherapeutic [22].

2.2. Coronary Artery Disease. The prevalence of OSA in patients with coronary artery disease is around 30% [23], while the prevalence among men hospitalized with acute myocardial infarction is nearly 70% [24]. Many characteristics of OSA can lead to cardiac ischemia such as intermittent hypoxia, sympathetic vasoconstriction and increased intrathoracic pressure. Sorajja et al. studied 200 subjects without a history of coronary artery disease and found the median coronary artery calcification score (Agatston units) was 9 in OSA patients compared to non-OSA patients. They also found that the median calcification score increased with the severity of OSA [25]. Another study shows that OSA patients are more likely to have a family history of premature death from coronary artery disease. Those results were independent of BMI, gender, and personal history of coronary artery disease [26]. However, a longitudinal analysis of data from the SHHS found that OSA was not a significant predictor of incidental CAD after adjustment for other risk factors [5].

2.3. Stroke. The prevalence of OSA in patients who have had a stroke is nearly 60% [27]. In a cross-sectional study of over 6,000 subjects from SHHS, the odds ratio of prevalent stroke was higher (1.58) in OSA patients with AHI ≥ 11 [28]. Several observational studies also show that OSA increases the chance of stroke incident [6, 29]. Redline et al. followed a total of 5,422 participants without a history of stroke at the baseline examination and untreated for OSA for a median of 8.7 years. A significant positive association between ischemic stroke and AHI was detected in men ($P = 0.016$). In OSA patients with AHI of 5–25, each unitary increase in AHI severity was associated with a 6% increase in stroke risk [30]. However, some factors rendered the relationship between OSA and stroke circumstantial. The population of patients who are at risk of stroke is demographically similar to the patients who are at risk of OSA. Also the fact that only survivors of stroke are tested complicates the causal association between stroke and OSA [31].

2.4. Heart Failure. The prevalence of OSA in HF ranges from 12% to 53% [32, 33]. Having OSA increases the mortality risk

Table 2: Different biomarkers of oxidative stress and ROS production in rodent models of intermittent hypoxia.

Reference	Species	Hypoxia regimen	Measured marker	Result
[8]	ICR mice	8 min cycles of FIO_2 8.5% and 21% for 30 days	MDA	Increased
[9]	$ApoE^{-/-}$ mice	30-s cycles of FIO_2 6.5%–21% 8 h/day for 4 and 12 weeks	OxLDL	Increased
[10]	C57BL/6J mice	2 min 6% O_2 and 2 min 21% O_2 for 8 h/day for 1, 2, and 4 weeks	TBARS	Increased
[11]	CF-1 mice	30-s cycles of FIO_2 8% 8 h/day for 21 and 35 days	DNA damage	Increased
[10]	C57BL/6J mice	2 min 6% O_2 and 2 min 21% O_2 for 8 h/day for 1, 2, and 4 weeks	Protein carbonyls	Increased
[12]	C57BL/6J mice	30-s cycles of FIO_2 4.5%–21% 8 h/day for 10 days	NADPH-dependent superoxide production	Increased
[13]	Sprague-Dawley rats	90-s cycles of FIO_2 of 5%, 20 cycle/h, 7 h/day for 14 days	Qualitative measurement of superoxide anion	Increased
[14]	Sprague-Dawley rats	21% to 10% FIO_2 for 5 s every 90 s for 4 weeks	SOD levels	Decreased

ApoE: apolipoprotein E, FIO2: fraction of oxygen inspired, MDA: malondialdehyde, NADPH: nicotinamide adenine dinucleotide phosphate, OxLDL: oxidized low-density lipoprotein, SOD: superoxide dismutase, TBARS: thiobarbituric acid.

of patients with ischemic HF, mainly because of sudden death [33]. Wang et al. showed that untreated OSA (AHI ≥ 15) in HF patients was associated with increased mortality rates when compared to (AHI ≤ 15) [34]. Gottlieb et al. reported that OSA predicted incident heart failure in men but not in women (adjusted hazard ratio 1.13 per 10-unit increase in AHI). Men with AHI > or = 30 were 58% more likely to develop heart failure than those with AHI < 5 [5]. OSA can lead to heart failure through many mechanisms including increases in blood pressure, left ventricular afterload, and by greater risk of myocardial infarction [35]. It is clear that OSA is related to many cardiovascular diseases and its treatment is a necessity.

3. Oxidative Stress in OSA

Oxidative stress results from an imbalance between antioxidant defense mechanisms and the production of oxidants; meaning that either a decreased antioxidant capacity and/or overproduction of reactive oxygen and nitrogen species (ROS/RNS) leads to a state of oxidative stress (Table 2). Although free radicals have important roles in regulating signal transduction and cellular function, their overproduction can damage lipids, proteins, and DNA, thus affecting many cellular and physiological mechanisms. Recent studies show important links between the hypoxia-related free radicals related oxidative stress and cardiovascular disease in OSA patients.

3.1. Sources of Reactive Oxygen Species in OSA. Free radicals are atoms or molecules containing one or more unpaired electrons in their atomic or molecular orbitals and thus are chemically unstable and highly reactive. Usually when two radicals react, the product is a nonradical, but when radicals react with nonradicals the product is a new radical and, therefore, the radical chain reaction propagates [36]. Oxygen metabolism during normal cellular respiration generates ROS as by-products, and their elimination occurs through enzymatic and nonenzymatic antioxidant systems. When ROS generation exceeds the capacity of antioxidants, oxidative stress and damage to cells and tissues ensue. This can contribute to pathological conditions of cardiovascular disease.

Oxygen has a unique electronic configuration; the addition of one electron to molecular oxygen can result in the production of the superoxide anion. Superoxide is considered the primary ROS and can interact with other molecules to generate secondary ROS/RNS, either directly or through enzyme- or metal-catalyzed processes. Superoxide anions can give rise to the production of many toxic molecules such as hydrogen peroxide, hydroxyl radical and peroxynitrite [37]. The latter is a RNS and results from a reaction between superoxide anion and nitric oxide, an important endothelium-derived vasodilator. As a result, nitric oxide bioavailability decreases and the vasodilator ability of blood vessels is compromised [38].

Mitochondria are major sources of superoxide anion due to reactions occurring during oxidative phosphorylation. It is estimated that 3–5% of the oxygen consumed by mitochondria is converted to superoxide anion during aerobic respiration. During hypoxia, ROS production is elevated due to excessive mitochondrial reduction [36]. NADPH oxidase is also a very important source of superoxide anion. Phagocytic cells contain this enzyme and other enzymes to produce ROS as a defense mechanism against pathogens. Although this mechanism can protect against invading microbes, it can also cause damage to surrounding tissue [39]. NADPH oxidase is also expressed in nonphagocytic cells where it usually generates lower amounts of superoxide anion for purposes such as signaling [40]. For example, NADPH oxidase is expressed in vascular cells where generation of superoxides plays an important role in vascular cell growth [41]. Figure 1 shows the production of different ROS, their physiologic function and role in disease state.

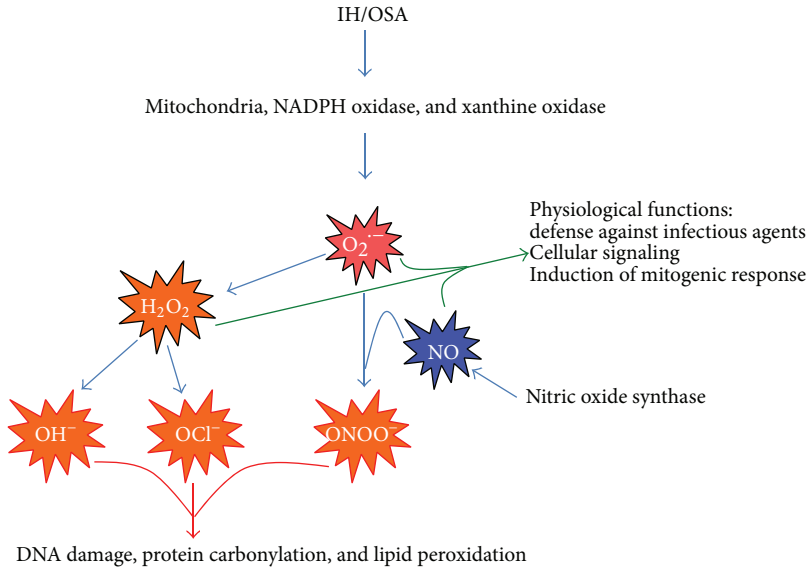

Figure 1: Reactive oxygen/nitrogen species produced during OSA/IH. H_2O_2: hydrogen peroxide, IH: intermittent hypoxia, NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase, NO: nitric oxide, $O_2^{\cdot-}$: superoxide anion, OCl^-: hypochlorite anion, OH^-: hydroxyl anion, $ONOO^-$: peroxynitrite, OSA: obstructive sleep apnea.

3.2. Evidence of Oxidative Stress in OSA. Many studies confirm the association of OSA with oxidative stress through measurements of oxidative stress markers. For example, Schulz et al. report increased production of superoxide anion in stimulated neutrophils and monocytes from OSA patients [42], while others report that superoxide anion production was significantly higher in nonstimulated monocytes of OSA patients [43, 44].

Oxidative stress markers of lipid peroxidation, protein carbonylation and DNA oxidation are increased in OSA patients. Lipid peroxidation is an important marker of oxidative stress since lipids are easily oxidized. Many studies show that lipid peroxidation increases in OSA patients. In an overnight study of OSA patients with and without cardiovascular disease, levels of thiobarbituric acid (TBARS), a marker of lipid peroxidation, were significantly increased [45]. In another study, fourteen males with severe OSA fasted all night and TBARS levels were measured in the next morning. TBARS levels in those patients were significantly higher (28.1 nmol MDA·mg^{-1} LDL protein) compared to thirteen healthy age matched controls (20.0 nmol MDA·mg^{-1} LDL protein) [46]. Oxidized LDL is also increased in OSA, where plasma levels of oxidized LDL were higher in OSA patients (43.6 U/L) compared to control (32.3 U/L) [47]. Protein carbonylation (oxidation of protein side chain) is increased as well in patients with moderate to severe OSA where protein carbonyl levels were significantly higher (1.11 μmol/g protein) when compared to matched controls (0.99 μmol/g protein). On the other hand, the increase was not significant in mild OSA patients (1.03 μmol/g protein) [48]. 8-hydroxyl-2′deoxyguanosine (8-OHdG), a marker of DNA oxidation, is also elevated in OSA patients. Urinary excretion of 8-OHdG significantly correlates with the severity of OSA [49].

Evaluating oxidative damage in OSA patients is essential since oxidative stress is one of the main causes of endothelial dysfunction. Yamauchi et al. studied 32 OSA and 15 control patients, in which they quantified endothelial nitric oxide synthase (eNOS), phosphorylated eNOS (the active form of the enzyme responsible for producing NO in the vasculature), inflammation (cyclooxygenase-2 and inducible NOS), and oxidative stress (nitrotyrosine). They also evaluated vascular reactivity in these patients by flow-mediated dilation [49]. Endothelial expression of eNOS and phosphorylated eNOS decreased by 59% and 94%, respectively, in untreated OSA patients. Nitrotyrosine and cyclooxygenase-2 expression was 5-fold greater in OSA patients. In patients who adhered to CPAP $\geq$ 4 hours a day, the expression of nitrotyrosine, cyclooxygenase-2, and inducible NOS was decreased significantly, while CPAP treatment restored eNOS and phosphorylated eNOS expression levels with concomitant reduction in oxidative stress. Of interest is that the effect of CPAP may be restricted to limiting free radical production, as antioxidant defense mechanisms were unaffected. Flow-mediated dilation in OSA patients was significantly decreased, but adhering to CPAP $\geq$ 4 hours a day significantly improved endothelial dependent vasodilation [50].

Antioxidant capacity is impaired in OSA patients. Although the antioxidant capacity in OSA subjects and controls did not differ in their study, Christou et al. showed a linear negative relationship between antioxidant capacity and apnea/hypopnea index ($R = -0.551$, $P = 0.041$) [51]. Total antioxidant status in OSA patients is significantly decreased (1.4 mmol/L) when compared to healthy subjects (1.5 mmol/L, $P = 0.0001$), with lower levels of vitamin A (64 μg/dL) and vitamin E (1525 μg/dL) when compared to control (74 and 1774 μg/dL, resp.) [52]. On the other hand, Katsoulis et al. reported some unexpected results where they found that total antioxidant status before and after sleep was significantly lower in OSA patients with AHI < 30 (1.73 versus 1.65 mmol/L, $P = 0.01$) but not in severe OSA patients with AHI > 30 (1.64

versus 1.58 mmol/L, $P = 0.07$). A possible explanation could be due to differences between the acute effects of hypoxia resulting from apneic sleep and chronic oxidative stress that may be sustained in severe OSA patients even during the daytime [53].

3.3. Oxidative Stress in Animals Subjected to Intermittent Hypoxia. OSA patients usually have comorbidities such as obesity, diabetes, or hypertension that likely will affect cause-effect relationships. Creating animal models of OSA would minimize the influence of comorbidities and behavioral variables common in humans. Using animal models also permits the use of pharmacological agents to study the pathological mechanisms under a well-controlled environment. Ideally, animal models should mimic OSA in humans in at least three ways: (a) they share aspects of the underlying pathophysiology, (b) have similar symptoms and the spectrum of disease severity that occur in humans, and (c) respond to treatment modalities that are useful in humans. Furthermore, a short life span (to allow for the unveiling of a wide range of disease-related complications within a reasonable time period), routine availability, cost effectiveness, and availability of disease-free littermates add to the usefulness of animal models. There are additional considerations when using animals that need to be considered for sleep-related research. Animal models for studying sleep-disordered breathing should address at least one (or a combination) of the three main injurious consequences of sleep apnea: intermittent hypoxia/hypercapnia, strained breathing due to mechanical obstruction, and sleep fragmentation. In this regard, rodents are amenable to genetic manipulation suitable for the production of phenotypes that may characterize OSA in humans. One advantage of using rodent models to examine neurophysiological aspects of sleep apnea in humans is the high degree of similarity between the structures of the nervous systems of rodents, such as rats and mice and humans.

A useful animal model of OSA is the English bulldog, since no surgical interventions or genetic manipulations are required. There is a strong resemblance in sleep apnea between humans and English bulldogs, making this animal model a suitable candidate for experimental use. It was noticed that these dogs snore and have hypopneas and frequent arousals from sleep, mainly due to an abnormal upper airway anatomy characterized by an enlarged soft palate and a narrowing of the oropharynx. These animals have episodes of both central and obstructive apnea with hemoglobin desaturation (<90%) that worsens during rapid eye movement (REM) sleep and is accompanied by daytime hypersomnolence as evidenced by a shortened sleep latency [54].

Alterations in the contractility of respiratory muscles were first reported in genetically obese Zucker fat rats (ZFR) in 1996 [55]. These animals show many of the cardiopulmonary deficits described in obese humans, such as respiratory control dysfunction, chest wall limitation, upper airway narrowing, hypertension, myocardial hypertrophy, and poor exercise capacity [56–58]. Later studies suggested that these rats also exhibit signs of sleep apnea [59].

OSA can also be stimulated through surgical procedures that induce airway obstruction [60]. This procedure has mainly been used in larger animals such as dogs Katayama et al. and Kimoff et al. [60, 61], piglets [62], baboons [63], and small rodents [64, 65]. Studies have incorporated sophisticated apparatus to detect sleep-wake states so that initiation of airway obstruction could be coordinated with sleep onset [66].

The most commonly used animal model in the area of OSA is the intermittent hypoxia (IH) model. This murine model represents extreme physiological changes occurring during sleep-related IH and was first described in 2001 by Tagaito et al. [67]. Mice are housed in customized cages to deliver either an intermittent hypoxic stimulus or an intermittent room air control. Ports evenly spaced near the bottom of the cages allow gases to enter from four sides at the level of the bedding material. A gas control delivery system regulates the flow of room air, N_2, and O_2 into the customized cages housing the mice. Programmable solenoids and flow regulators control the manipulation of inspired O_2 fraction (FI_{O_2}) levels in each cage over a wide range of IH profiles. During the 12-h light cycle, FI_{O_2} is reduced from 20.9 to 5.0% over a 30-s period and rapidly reoxygenated to room air levels using a burst of 100% O_2 during the following 30-s period. During the 12-h dark cycle, a constant flow of room air is delivered to the cages. The use of multiple inputs into the cage produces a uniform nadir FI_{O_2} level throughout the cage.

There is much support in the literature for the idea that oxidative stress is a consequence of intermittent hypoxia. Rats subjected to intermittent hypoxia for two weeks have increased vascular production of ROS [13]. IH-induced pulmonary hypertension in mice leads to increased lung levels of the NADPH oxidase subunits NOX4 and p22phox, indicating that NADPH oxidase-derived ROS contributes to the development of pulmonary hypertension caused by chronic intermittent hypoxia [68]. NADPH oxidase is activated in tissues such as the myocardium, brain, carotid body, and liver in various animal models of IH [69–71]. As for oxidative stress markers, one month of IH significantly increased MDA levels in mice [8]. This is in agreement with a study by Savransky et al. who reported that serum MDA levels increased 4-fold in mice subjected to chronic IH for 6 months when compared to control [72]. Oxidative stress markers are also elevated in tissues such as the liver and brain Rosa et al. and Xu et al. [11, 73].

3.4. Oxidative Stress Causes Endothelial Dysfunction in OSA. Diminished endothelial function is an important consequence of OSA and is frequently measured as impaired endothelium dependent vasodilatation [74]. Eventually endothelial dysfunction leads to atherosclerosis, a condition where artery walls become narrow due to the buildup of fatty materials, cholesterol, macrophages, cellular debris, and other substances. These changes create significant reductions in blood flow through the affected artery [75]. Although the etiology of atherosclerosis is unknown, several factors such as elevated levels of LDL, low levels of HDL, hypertension, diabetes mellitus, male gender, obesity, family history, infectious disease, and environmental factors are implicated. Many of these factors lead to endothelial dysfunction and atherosclerosis through a unifying mechanism of oxidative stress

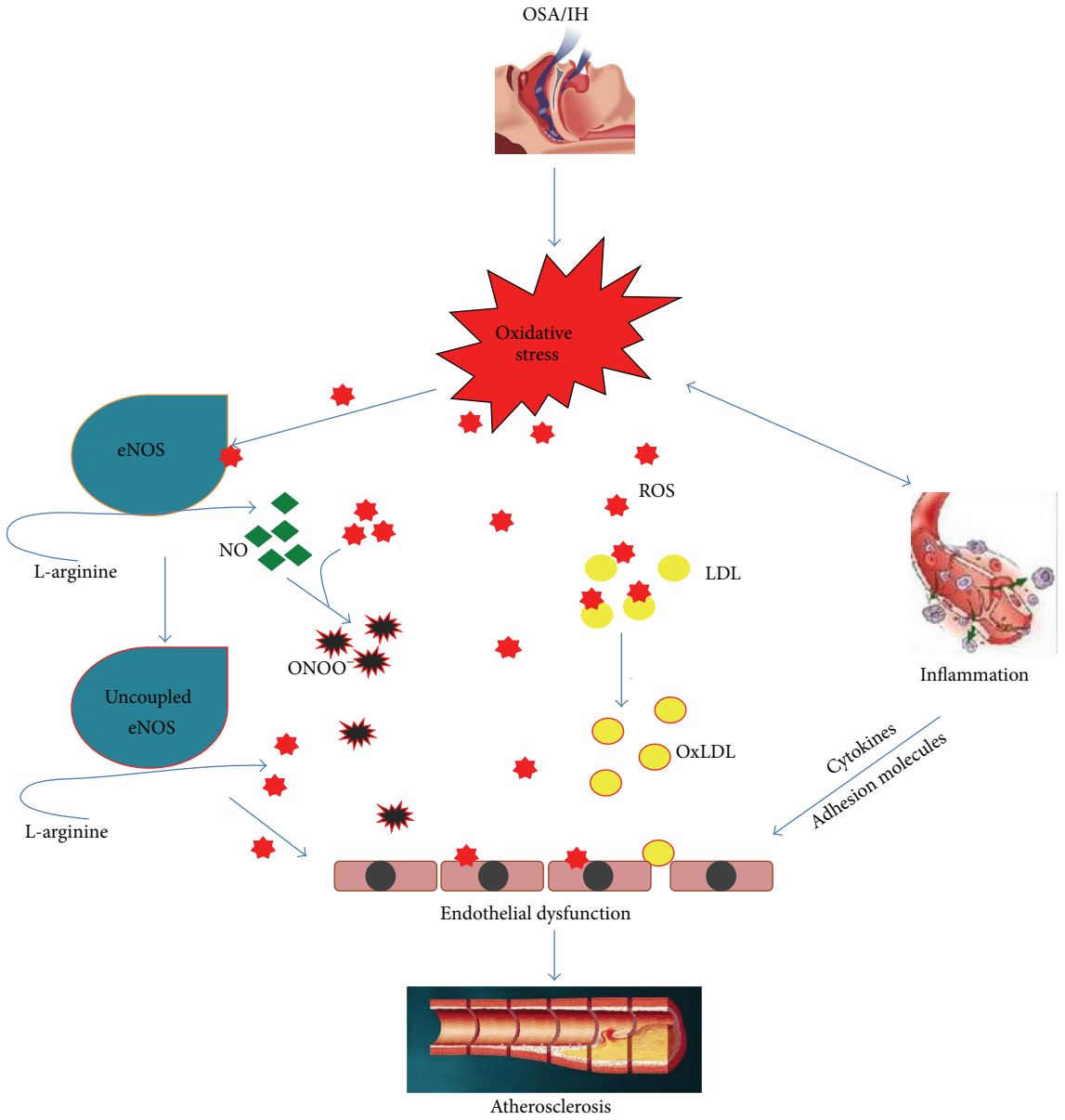

Figure 2: OSA/IH can lead to oxidative stress, which through many mechanisms can cause endothelial function, which eventually progresses to atherosclerosis. eNOS: endothelial nitric oxide synthase, IH: intermittent hypoxia, LDL: low density lipoprotein, NO: nitric oxide, $ONOO^-$: peroxynitrite, OSA: obstructive sleep apnea, OxLDL: oxidized low density lipoprotein, ROS: reactive oxygen species.

and inflammation [76]. Various studies show lower levels of circulating NO in OSA, for example, by the reduced levels of serum nitrite/nitrate (by-products of normal NO metabolism) in OSA subjects (38.9 μM versus 63.1 μM in controls) [77]. This was confirmed in other studies where nitrate/nitrite levels were significantly lower in OSA patients (35.6 μM) when compared to control (72.6 μM) [78]. Many mechanisms have been suggested for endothelial dysfunction due to OSA or IH including (1) interaction on NO and ROS forming peroxynitrite, (2) uncoupling of eNOS, and (3) decreased endothelial expression of eNOS and increased levels of endogenous eNOS inhibitors [42]. Due to its short half-life and large volume of distribution, peroxynitrite is hard to measure and these factors explain the lack of difference in nitrotyrosine levels between OSA and healthy subjects [79, 80]. However, Jelic and Le Jemtel found an increased expression of nitrotyrosine in endothelial cells derived from OSA patients [50].

In all the forms of nitric oxide synthase, including the endothelial one, enzymatic activity requires five cofactor groups to incorporate oxygen into the amino acid L-arginine to produce NO. Those cofactors are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4), and Ca^{2+}-calmodulin. If nitric oxide synthase lacks L-arginine or another of the necessary cofactors, it will produce superoxide anion instead of NO through an uncoupled state of nitric oxide synthase [81]. Antoniades et al. reported that increased ROS production

during hypoxia could lead to BH_4oxidation and increased levels of arginase II that degrades L-arginine, leading to further eNOS uncoupling [82]. Patients with OSA have increased levels of asymmetrical dimethylarginine (ADMA), a competitive inhibitor of NOS [83]. Studies by Tanaka et al. suggest that eNOS activation is sensitive to regulation by redox status and that oxidative stress leads to decreased eNOS phosphorylation, so reducing its activity [84], while Jelic and Le Jemtel supported the latter findings when they reported decreased ratios of total phosphorylated eNOS in endothelial cells from OSA [50]. Figure 2 explains how OSA can lead to atherosclerosis through oxidative stress mediated mechanisms.

3.5. Oxidative Stress and Heart Failure in OSA. Almost 20–40% of OSA patients have mild pulmonary hypertension even in the absence of lung disease or left-sided heart disease [85–87]. Severe OSA patients also seem to have abnormalities of right ventricular function [88–90]. The clinical significance of pulmonary pressure changes and right ventricular function is uncertain, and it is not known whether they are sufficient to progress to right ventricular failure in the absence of other cardiopulmonary diseases. It seems that OSA can lead also to left ventricular heart failure when there are comorbidities such as chronic lung disease, obesity, or left ventricular failure [91].

During hypoxic conditions, pulmonary artery constricts mainly due to the ability of their smooth muscles to sense changes in O_2. An increase in pulmonary vascular resistance exerts a pressure overload to the right ventricle, resulting in hypertrophy followed by dilated cardiomyopathy [92]. In addition to the pulmonary artery, the carotid body can also sense changes in O_2. It is well established that oxygen sensing by the carotid body plays an important role in the development of systemic hypertension associated with intermittent hypoxia and OSA [93]. The molecular mechanisms of oxygen sensing in these arteries involve ROS-induced closure of K^+ channels which might be responsible for the acute changes in response to altered oxygen tension [94].

4. Summary

Obstructive sleep apnea is an independent risk factor for cardiovascular disease. It is well accepted that intermittent hypoxia in OSA resembles hypoxia/reperfusion injury mechanisms responsible for ROS overproduction. Intermittent hypoxia can promote endothelial dysfunction and heart disease through oxidative stress. However, more research is needed to increase our understanding of the mechanisms that induce cardiovascular disease in OSA and so leads to new and more effective treatment modalities to prevent the cardiovascular risks associated with this increasingly common disease.

Conflict of Interests

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

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Research Article

Plasma Levels of Aminothiols, Nitrite, Nitrate, and Malondialdehyde in Myelodysplastic Syndromes in the Context of Clinical Outcomes and as a Consequence of Iron Overload

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The role of oxidative stress in the initiation and progression of myelodysplastic syndromes (MDS) as a consequence of iron overload remains unclear. In this study we have simultaneously quantified plasma low-molecular-weight aminothiols, malondialdehyde, nitrite, and nitrate and have studied their correlation with serum iron/ferritin levels, patient treatment (chelation therapy), and clinical outcomes. We found significantly elevated plasma levels of total, oxidized, and reduced forms of cysteine ($P < 0.001$), homocysteine ($P < 0.001$), and cysteinylglycine ($P < 0.006$) and significantly depressed levels of total and oxidized forms of glutathione ($P < 0.03$) and nitrite ($P < 0.001$) in MDS patients compared to healthy donors. Moreover, total ($P = 0.032$) and oxidized cysteinylglycine ($P = 0.029$) and nitrite ($P = 0.021$) differed significantly between the analyzed MDS subgroups with different clinical classifications. Malondialdehyde levels in plasma correlated moderately with both serum ferritin levels ($r = 0.78$, $P = 0.001$) and serum free iron levels ($r = 0.60$, $P = 0.001$) and were significantly higher in patients with iron overload. The other analyzed compounds lacked correlation with iron overload (represented by serum iron/ferritin levels). For the first time our results have revealed significant differences in the concentrations of plasma aminothiols in MDS patients, when compared to healthy donors. We found no correlation of these parameters with iron overload and suggest the role of oxidative stress in the development of MDS disease.

1. Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematological disorders, characterized by ineffective hematopoiesis and a high risk of transformation into acute myeloid leukemia (AML). Although the origin of MDS development is not fully understood, it has been determined that oxidative stress plays an important role in the initialization and disease progression of MDS [1].

One of the suggested mechanisms causing oxidative stress in MDS is attributed to a non-transferrin-bound iron (NTBI or free iron), which has been found in higher levels in the early stages of MDS patients receiving frequent red blood cell (RBC) transfusions [2]. Several studies have found elevated levels of oxidative stress markers (reactive oxygen species) and reduced levels of antioxidants (reduced glutathione (GSH)) in MDS patients and their correlation with serum ferritin levels [3, 4]. However, increased oxidative stress was revealed, even in the patients not receiving transfusions [5]. The presence of several other oxidative stress markers has been described in patients with established MDS, independent of iron or ferritin levels [6–8].

Oxidative stress, the imbalance in prooxidative and antioxidative processes, in favour of the first, acts through reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative status is reflected in blood plasma by actors of oxidative stress (free radicals and their metabolites), their products such as modified biomacromolecules,

products of lipid peroxidation (malondialdehyde (MDA), 4-hydroxynonenal), and changes in the concentrations of compounds involved in antioxidant defense (enzymes, macromolecular and low-molecular-weight antioxidants, e.g., aminothiols).

Oxidative stress has been related to the origin and progression of a growing number of human diseases; however, their clear correlation is far from being proven [9]. Key factors influencing the evaluation of oxidative stress and its relation to the disease pathogenesis have been pointed out. They are (1) the choice of biomarker(s) and/or the biological system(s) for the analyses; (2) pitfalls in preanalytical and analytical methods for assessing oxidative stress; and (3) scientific misconduct [9]. Considering discussed factors simultaneous determination of plasma oxidative stress actors, their products, and antioxidant defense molecules is necessary to investigate the role of oxidative stress in the pathogenesis of MDS. The only work simultaneously evaluating oxidative stress markers and antioxidant defense molecules was done by Ghoti et al. in blood cells; however, to the best of our knowledge we have not found any work evaluating oxidative stress markers and antioxidant defense molecules in plasma of MDS patients and their relationships with each other and with iron and ferritin levels.

The aim of this study has been to assess the oxidative status of MDS patients and healthy donors by the evaluation of levels of antioxidant defense molecules (plasma total, oxidized, and reduced forms of aminothiols: GSH, cysteine (Cys), cysteinylglycine (CG), and homocysteine (Hcys)), marker of oxidative stress (MDA), and metabolites of NO (nitrite (NO_2^-) and nitrate (NO_3^-)) Although plasma nitrite and nitrate are not significant biomarkers of oxidative stress, they reflect NO species in plasma. We further estimated their relationship with serum iron/ferritin levels and clinical outcomes in MDS patients.

2. Materials and Methods

2.1. Materials and Reagents. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. All reagents employed were of analytical grade or higher purity, and all aqueous solutions were prepared using HPLC-grade water.

2.2. Blood Plasma Samples. Blood samples were retrospectively collected from 61 patients with MDS, diagnosed at the Institute of Hematology and Blood Transfusion, Prague, Czech Republic, and from 23 healthy volunteers. None of the patients had received any specific therapeutic agents prior to the study. Patients were not on any special diet prior to the study. All individuals tested agreed to the study at the time of blood collection. All samples were obtained in accordance with the Ethical Committee regulations of the Institute of Hematology and Blood Transfusion, Prague; and with a release of informed consent. Blood samples were drawn from patients and controls in a vacutainer tube containing EDTA for plasma, or containing beads coated with a clotting activator for serum (serum iron and serum ferritin determination); the tubes were immediately cooled on an ice bag and centrifuged as soon as possible at 4000 ×*g*, for 5 min, at 4°C. Serum and plasma samples were stored in the dark at −70°C until the analysis.

Diagnoses were made according to the 2008 WHO and FAB classification systems. Patients with unclassified MDS, Fanconi anemia, chronic myeloid leukemia, autoimmune thrombocytopenia, and congenital anemia have been excluded from the analysis. Thus, the following categories were considered: refractory anemia (RA), MDS with isolated del(5q) (RA-5q), refractory anemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), RCMD with ringed sideroblasts (RCMD-RS), RCMD with 5q deletion (RCMD-5q), RCMD with reactive monocytes, RA with an excess of blasts-1 (RAEB-1), RA with an excess of blasts-2 (RAEB-2), RA with an excess of blasts in transformation (RAEB-T), myelodysplastic/myeloproliferative syndromes (MDS/MPS), MDS/MPS with 5q deletion, acute myeloid leukemia (AML M2), and MDS-RAEB1 plus SC-non-Hodgkin lymphoma. In our study there were 19 patients with cardiovascular event and 28 patients with hypertension.

All the healthy subjects enrolled in this study were asymptomatic and none of them had any abnormality on physical examination and routine blood laboratory tests. No one was taking medication, smoked, or drank alcohol, and all gave informed written consent before participating in this study.

2.3. Ferritin, Iron, and Gamma-Glutamyltransferase. Serum ferritin, serum iron (Fe), and gamma-glutamyltransferase (GGT) plasma levels were estimated in healthy controls and MDS patients in the Central National Biochemical Laboratory in the Institute of Hematology and Blood Transfusion. Values of ferritin, iron, and GGT in healthy donors fall within the limits of the reference interval.

2.4. Measurement of Total and Reduced Forms of Thiols (Cys, Hcys, GSH, and CG). Plasma samples were treated according to Raijmakers et al. with several modifications [10]. 60 μL of plasma sample or standards was mixed with 60 μL of PBS and 15 μL of 10% tris(2-carboxyethyl) phosphine (w/v) for total levels of thiols (sum of their reduced and oxidized forms in plasma, including thiols covalently bound to plasma proteins) or with 75 μL of PBS for reduced forms of thiols. Both mixtures were incubated at 25°C for 30 min and deproteinized by the addition of 135 μL of 10% trichloracetic acid with 2 mM EDTA, followed by centrifugation (15000 ×*g*, 15 min, 10°C). To 50 μL of supernatant, 125 μL of 125 mM borate buffer (pH 9.5) with 4 mM EDTA was added, followed by the addition of 15 μL of 1.5 M NaOH and 50 μL of 0.1% ammonium 7-fluorobenzofurazan-4-sulfonate (w/v). The reaction mixture was incubated at 60°C in darkness for 60 min and filtered through 0.2 μm cellulose filters (National Scientific, Rockwood, TN, USA). Chromatographic conditions were used according to Garcia [11], with several modifications using a High Performance Liquid Chromatographic system (HPLC) (Shimadzu, Tokyo, Japan). A 20 μL aliquot was injected into

a LUNA C18 (2) column (150 × 3 mm, 5 μm) (Phenomenex, Torrance, CA, USA) and separated at 40°C. The mobile phase was composed of 0.1 M H_3PO_4 adjusted to pH 2.1 with KOH (A) and 10% methanol buffer A (B). Elution of the thiol-benzofurazan-4-sulfonate derivatives was performed with a gradient system (t (min)/% B: 0/0, 7/0, 7.1/100, 10/100, 10.1/0) at a flow rate of 1 mL/min. The fluorescence signal of excitation was measured at 385 nm and of emission at 515 nm.

2.5. Measurement of Nitrite. Nitrite standards and samples were prepared as previously described by Li et al. [12]. Briefly, 200 μL of nitrite standard KNO_2 (0–625 nM) or a patient sample (10x diluted and ultrafiltered plasma sample) was incubated at 25°C with 20 μL 316 mM 2,3-diaminonaphthalene (in 0.62 M HCl) for 10 min, followed by the addition of 8 μL of triethylamine. The dilution of the sample with water could influence real concentrations of nitrite in plasma with contaminating nitrite in water. This reaction mixture was deproteinized with acetonitrile 1 : 1, centrifuged (17000 ×g, 4 min), and filtered through a 0.2 μm cellulose filter (National Scientific). 150 μL of the filtered solution was used directly for the chromatographic separation of reaction product 2,3-naphthotriazole. Chromatographic conditions were used as described by Woitzik et al. [13] with minor changes using a HPLC system (Shimadzu). A 10 μL aliquot of the sample was injected into a Luna C18 (2) column (150 × 3 mm, 5 μm) (Phenomenex). The mobile phase consisted of 30% acetonitrile in 30 mM of phosphate buffer, adjusted to pH 8 with triethylamine. Fluorescence was monitored with excitation at 375 nm and emission at 415 nm, with separations performed at 45°C.

2.6. Measurement of Nitrate. Nitrate was determined according to Davies et al. [14] by capillary electrophoresis with UV detection at 214 nm (Beckman Coulter, Fullerton, CA, USA), performed at 25°C with small changes. The components were separated using a –10 kV voltage; reverse electroosmotic flow was used. The separation of samples took place in a fused silica capillary tube (50 μm diameter by 40 cm to the detector), in a buffer consisting of 150 mM NaCl/5 mM Tris-HCl (pH 7.4) and 2 mM tetradecyltrimethylammonium hydroxide (TTAH). TTAH was prepared from a tetradecyltrimethylammonium bromide solution by passing it through a strong anion exchange cartridge (Phenomenex), which replaced the bromide ions with hydroxide ions. The capillary tube was rinsed before each injection with 0.1 M NaOH and a separation buffer for 1 min and 2 min, respectively. Plasma samples were centrifuged (17000 ×g, 4 min, 25°C), filtered through a 0.2 μm cellulose filter (National Scientific), and sonicated. The linearity of the assay was determined by preparing aqueous solutions containing 0.39 μM–500 μM KNO_3.

2.7. Measurement of MDA. Standard and plasma samples were prepared as in our previous study [15]. Briefly MDA standards were prepared by adding 25 μL tetrahydroxypropane into 50 mL of 1% H_2SO_4 (v/v). Mixture was incubated in darkness for 2 hours. Concentration of MDA was measured spectrophotometrically (ε 245 nm = 13700 $cm^{-1} \cdot L \cdot mol^{-1}$). 100 μL of a plasma or standard sample (0–10 μM) was mixed with 12.5 μL of 100 mM EDTA in 2% NaOH (w/v), 12.5 μL of H_2O or MDA standards, and 125 μL of 10 mM 2,6-ditert-butyl-4-methylphenol in acetonitrile. The mixture was incubated at 60°C for 30 min. Samples were centrifuged (17000 ×g, 10 min). To 75 μL of the supernatant, a total of 300 μL of 25 mM 2-thiobarbituric acid in 2 M CH_3COOH was added (pH 3) and incubated at 100°C for 60 min. Separations were carried out on a 5 μm reversed-phase C18 Gemini NX column (150 × 2 mm) (Phenomenex) at 25°C using a HPLC system (Shimadzu), as was described in our previous work [15]. Elution of the MDA derivative with 2-thiobarbituric acid was performed isocratically with 35% MeOH in 50 mM of NH_4HCO_3 buffer, adjusted to pH 9.3 with NH_4OH at a flow rate of 0.25 mL/min, with UV-Vis detection at 532 nm.

2.8. Statistical Analysis. Data are presented as means ± standard deviation (SD) and as a range. A two-tail-, -two sample Student's t-test was used to compare MDS patients with healthy donors. One-way ANOVA was computed to examine the differences across all groups (MDS, healthy controls). Post hoc analyses using Duncan homogeneous subsets were performed for the cases in which the main effect was significant. A Pearson correlation test was used for the normally distributed data and a Spearman's rank correlation test for nonparametric data. All tests for statistical significance were standardized at an alpha level of $P < 0.05$.

All methods used were performed according to standard operating procedures (SOP) validated and verified. The methods have been optimized and validated for selectivity, precision, and recovery using an internal quality control. All of the tested compounds analyzed by chromatography methods had linearity of >98%, with relative standard deviation <10% in terms of variation of retention time. Interday and intraday variability was <5%.

3. Results

3.1. Oxidative Stress Parameters in MDS Patients and Healthy Donors. Plasma levels of total, oxidized, and reduced forms of aminothiols Cys, Hcys, and CG were elevated in the plasma of all MDS patients, when compared with healthy controls using a two-tailed, two-sample Student's t-test. Conversely, plasma levels of total (t-GSH) and oxidized (ox-GSH) forms of GSH and nitrite were significantly depressed in all MDS patients, comparing with healthy donors. We did not observe any significant differences between MDS patients and healthy donors in plasma levels of MDA and nitrate. Serum ferritin levels exceeded the upper limit of the reference interval in MDS patients. The means of free iron serum levels were in the reference interval and levels of GGT were at the upper limit edge of the reference interval in all MDS patients. Data are shown in Tables 1 and 2.

3.2. Oxidative Stress Parameters in MDS Patients in the Context of Clinical Outcomes. Subsequently, MDS patients

Table 1: Plasma levels of oxidative stress compounds in healthy donors and myelodysplastic patients (MDS). Data are expressed as means ± SD. Significant differences between MDS patients and healthy donors are marked with ∗. Data were analyzed using a two-tailed, two-sample Student's t-test.

Analyzed compounds	Healthy donors ($n = 23$)	MDS patients ($n = 61$)
t-Cys (μM)	219 ± 14	284 ± 68***
t-Hcys (μM)	9.45 ± 0.63	14.50 ± 10.70***
t-CG (μM)	39.71 ± 4.86	48.80 ± 14.30**
t-GSH (μM)	9.07 ± 1.55	7.16 ± 3.59*
MDA (μM)	0.69 ± 0.11	0.82 ± 0.34
NO_2^- (nM)	1149 ± 86	903 ± 215***
NO_3^- (μM)	32.78 ± 10.33	32.80 ± 17.87
Ferritin (μg/L)	Ref. R.: 22–322	557.9 ± 4.0
Fe (μM)	Ref. R.: 7.2–29	26.15 ± 12.28
GGT (μkat/L)	Ref. R.: 0.14–0.8	0.60 ± 0.56

$^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P < 0.05$, total cysteine (t-Cys), total homocysteine (t-Hcys), total cysteinylglycine (t-CG), total glutathione (t-GSH), malondialdehyde (MDA), and gamma-glutamyltransferase (GGT).

Table 2: Plasma levels of total, reduced, and oxidized forms of thiols in healthy donors and myelodysplastic patients (MDS). Data are expressed as means ± SD. Significant differences between MDS patients and healthy donors are marked with ∗. Data were analyzed using a two-tailed, two-sample Student's t-test.

Analyzed compounds	Healthy donors ($n = 23$)	MDS patients ($n = 61$)
t-Cys (μM)	219 ± 14	284 ± 68***
red-Cys (μM)	4.06 ± 1.07	8.10 ± 3.46***
ox-Cys (μM)	215 ± 14	275 ± 67***
t-Hcys (μM)	9.45 ± 0.63	14.50 ± 10.70***
red-Hcys (μM)	0.03 ± 0.01	0.05 ± 0.07*
ox-Hcys (μM)	9.43 ± 0.63	14.45 ± 10.64***
t-CG (μM)	39.71 ± 4.86	48.8 ± 14.30**
red-CG (μM)	1.50 ± 0.51	3.35 ± 2.33***
ox-CG (μM)	38.21 ± 4.57	45.44 ± 12.72*
t-GSH (μM)	9.07 ± 1.55	7.16 ± 3.59*
red-GSH (μM)	0.39 ± 0.09	0.41 ± 0.39
ox-GSH (μM)	8.68 ± 1.48	6.74 ± 3.26*

$^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P < 0.05$, total cysteine (t-Cys), reduced cysteine (red-Cys), oxidised cysteine (ox-Cys), total homocysteine (t-Hcys), reduced homocysteine (red-Hcys), oxidised homocysteine (ox-Hcys), total cysteinylglycine (t-CG), reduced cysteinylglycine (red-CG), oxidised cysteinylglycine (ox-CG), total glutathione (t-GSH), reduced glutathione (red-GSH), and oxidised glutathione (ox-GSH).

were divided into four study groups (1–4) according to their common clinical and diagnostic outcomes (Table 3). The groups were compared with each other and with a group of healthy donors (0). Table 3 provides an overview of the groups analyzed in the presented study. ANOVA was used to test for significant differences in the means of measured compounds concentrations between the analyzed groups. As shown in Table 4, significant differences were observed between the analyzed groups for total CG (t-CG) ($P = 0.032$) and for nitrite ($P = 0.021$). Oxidized CG (ox-CG) also significantly differed between groups ($P = 0.029$). Using post hoc ANOVA tests (Duncan) we ascertained that the levels of t-CG (Figure 1) and ox-CG were significantly higher in group 1 of MDS patients, with respect to the healthy donors and group 4. The same post hoc test showed the levels of nitrite significantly lower in all MDS subgroups as compared to the healthy controls (Figure 2).

Plasma levels of all forms of Cys were also higher in all MDS subgroups, as compared to the healthy donors. The highest plasma levels were found mostly in groups 1 and 2, respectively. Total, oxidized, and reduced GSH concentrations tended to be lower in all MDS patients, as compared to the healthy donors; however, there was not a statistical significance between the groups. The levels of MDA were higher in groups 1 and 3, as compared with healthy donors; but these data differences were also not statistically significant. Plasma levels of nitrate in groups 0, 1, and 2 were approximately the same; lower values were observed in groups 3 and 4, with the lowest value in group 4; yet they still did not differ significantly. Levels of serum free iron and serum ferritin did not differ significantly between the MDS groups.

Using a Pearson correlation test, a moderate positive correlation was found between all forms of evaluated thiols. Moreover, reduced Cys (red-Cys) correlated moderately with t-CG ($r = 0.40$, $P = 0.001$) and moderately with reduced CG (red-CG) ($r = 0.77$, $P = 0.001$). A moderate correlation between the levels of t-CG and t-GSH ($r = 0.65$, $P = 0.001$) (Figure 3) and moderate correlation between both ox-CG ($r = 0.48$, $P = 0.001$) and red-CG ($r = 0.39$, $P = 0.001$) and t-GSH was found. Moreover, a moderate negative correlation was found between the concentrations of all forms of CG: t-CG ($r = -0.41$, $P = 0.005$), ox-CG ($r = -0.40$, $P = 0.001$), and red-CG ($r = -0.49$, $P = 0.001$), and nitrite (Figure 4).

3.3. Oxidative Stress Parameters in MDS Patients in the Context of Iron Overload. Patients were divided according to the possible risk of iron overload (high iron and ferritin levels) into a group of patients requiring chelation therapy (CH) (16) and a group of patients not requiring chelation therapy (non-CH) (30). Applying a two-tailed, two-sample Student's t-test, we estimated that the levels of serum free iron ($P = 0.001$) and serum ferritin ($P = 0.006$) were significantly higher in the CH group. However, no significant differences in aminothiol, nitrite, and nitrate levels were found between these two groups. MDA concentrations were significantly higher in the group CH ($P = 0.001$). Furthermore, we compared analyzed compounds in patients regularly receiving blood transfusions (T) with patients who were not receiving blood transfusions (non-T). We found significantly higher levels of serum free iron ($P = 0.004$) and MDA ($P = 0.013$) in the group T of patients. No significant differences were found between the groups for the other analyzed compounds.

Table 3: Overview of the analyzed study groups (0–4), the number of myelodysplastic patients, gender ratio, and the age of analyzed patients and controls.

Group	Number of patients (male/female)	Age range	Diagnoses
0	23 (10/13)	25–57	Healthy donors
1	14 (10/4)	52–91	RA, RA-5q, RARS
2	29 (15/14)	28–90	RCMD, RCMD-RS, RCMD-5q, RCMD with reactive monocytes
3	9 (4/5)	36–85	RAEB-1, MDS/MPS, MDS/MPS with 5q deletion, MDS-RAEB-1 + SC-NHL
4	9 (5/4)	55–80	RAEB-2, RAEB-T, AML M2

Refractory anemia (RA), MDS with isolated del(5q) (RA-5q), refractory anemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), RCMD with ringed sideroblasts (RCMD-RS), RCMD with 5q deletion (RCMD-5q), RCMD with reactive monocytes, RA with an excess of blasts-1 (RAEB-1), RA with an excess of blasts-2 (RAEB-2), RA with an excess of blasts in transformation (RAEB-T), myelodysplastic/myeloproliferative syndromes (MDS/MPS), MDS/MPS with 5q deletion, acute myeloid leukemia (AML M2), and MDS-RAEB-1 plus SC-non-Hodgkin lymphoma.

Table 4: Oxidative stress parameters in the control group (0) and four myelodysplastic syndromes subgroups (1–4). Values are shown as means ± SD. Data were analyzed by ANOVA, and P values are shown for significantly differing parameters.

Analyzed compounds	Analyzed groups					P value
	0	1	2	3	4	
t-Cys (μM)	219 ± 14	300 ± 57	284 ± 71	274 ± 56	268 ± 90	
t-Hcys (μM)	9.45 ± 0.63	11.73 ± 4.87	17.12 ± 14.51	13.50 ± 3.17	11.38 ± 4.75	
t-CG (μM)	39.71 ± 4.86	56.09 ± 18.81	47.54 ± 11.41	49.70 ± 8.15	40.59 ± 14.95	*0.032
t-GSH (μM)	9.07 ± 1.55	8.36 ± 4.27	6.83 ± 2.88	7.25 ± 4.89	6.26 ± 3.22	
MDA (μM)	0.70 ± 0.12	0.99 ± 0.51	0.74 ± 0.25	0.92 ± 0.31	0.71 ± 0.15	
NO_2^- (nM)	1185 ± 93	960 ± 275	890 ± 225	827 ± 108	945 ± 173	*0.021
NO_3^- (μM)	34.80 ± 11.16	33.14 ± 13.87	37.47 ± 20.32	29.29 ± 18.91	20.78 ± 6.91	
Ferritin (μg/L)	Ref. R.: 22–322	1569 ± 2	330 ± 6	1681 ± 2	250 ± 3	
Fe (μM)	Ref. R.: 7.2–29	38.19 ± 9.13	25.23 ± 13.15	29.44 ± 7.20	19.51 ± 10.1	
GGT (μkat/L)	Ref. R.: 0.14–0.8	0.75 ± 0.77	0.70 ± 0.71	0.60 ± 0.17	0.42 ± 0.27	

Total cysteine (t-Cys), total homocysteine (t-Hcys), total cysteinylglycine (t-CG), total glutathione (t-GSH), malondialdehyde (MDA), and gamma-glutamyltransferase (GGT).

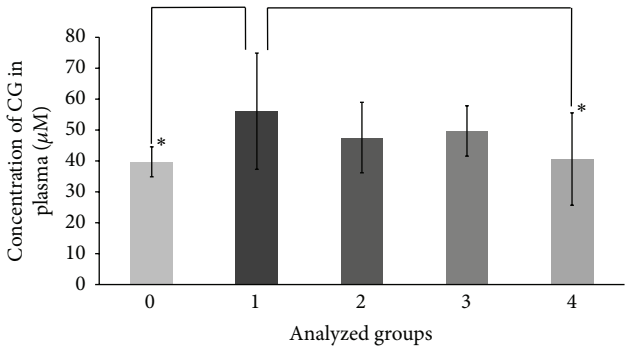

Figure 1: Concentration of total cysteinylglycine (t-CG). Plasma levels of t-CG (reduced form and form bound to proteins) in healthy donors (0) and MDS subgroups (1–4). Data are expressed as means ± SD. Using ANOVA, t-CG was found to differ significantly between groups ($P = 0.032$). *Statistical significance of the difference between group 1 and both groups 0 and 4 (post hoc ANOVA tests, Duncan, $P < 0.05$).

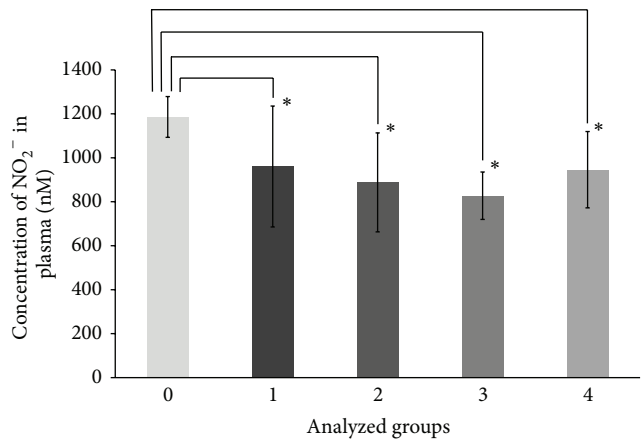

Figure 2: Concentration of nitrite (NO_2^-). Plasma levels of nitrite in healthy donors (0) and MDS subgroups (1–4). Data are expressed as means ± SD. Using ANOVA, nitrite was found to differ significantly between groups ($P = 0.021$). *Statistical significance of the difference between groups 1–4 and the control group (post hoc ANOVA tests, Duncan, $P < 0.05$).

Our data showed that while a lack of correlation was found between plasma oxidative stress parameters (aminothiols, nitrite, and nitrate) and serum iron levels/ferritin levels, MDA correlated moderately with both serum free iron levels ($r = 0.60$, $P = 0.001$) (Figure 5) and serum ferritin levels ($r = 0.78$, $P = 0.001$) (Figure 6).

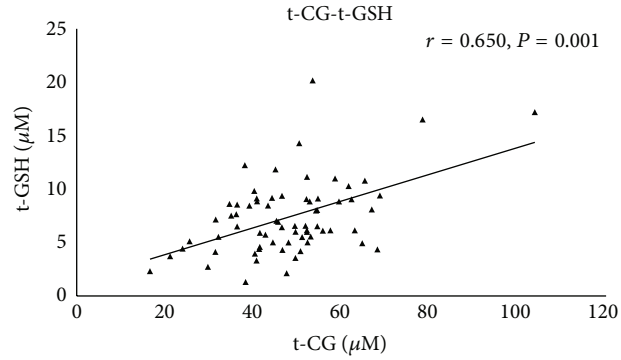

Figure 3: Correlation of total cysteinylglycine (t-CG) and total glutathione (t-GSH). Correlation of t-CG plasma concentrations and t-GSH plasma concentrations in MDS patients and healthy donors. *P* and *r* values were derived by a Pearson correlation test.

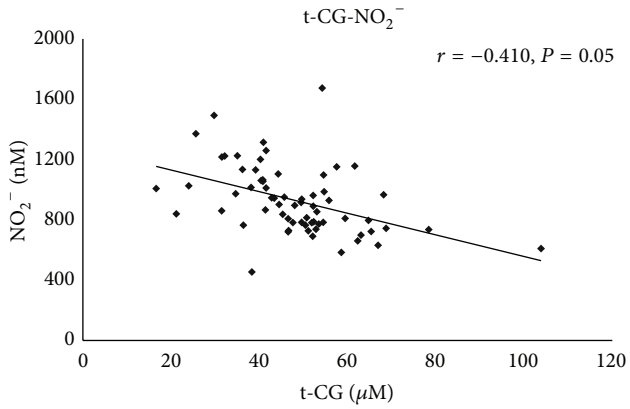

Figure 4: Correlation of cysteinylglycine (t-CG) and nitrite (NO_2^-). Correlation of t-CG plasma concentrations and nitrite plasma concentrations in MDS patients and healthy donors. *P* and *r* values were derived by a Pearson correlation test.

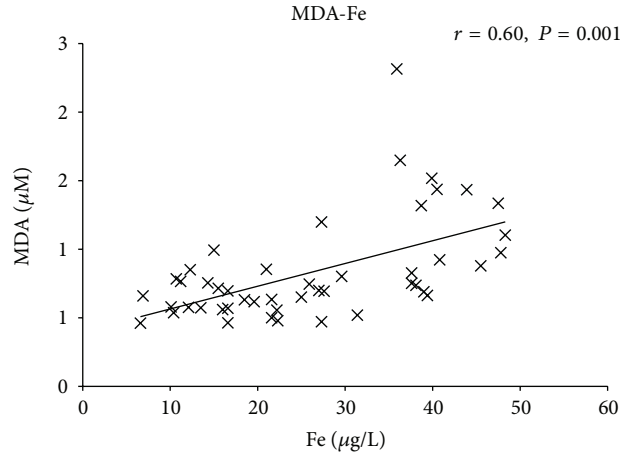

Figure 5: Correlation of total malondialdehyde (MDA) and free iron (Fe). Correlation of MDA plasma concentrations and Fe serum concentrations in MDS patients and healthy donors. *P* and *r* values were derived by a Pearson correlation test.

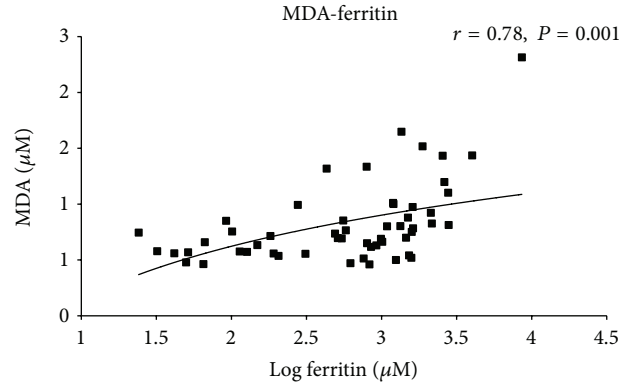

Figure 6: Correlation of total malondialdehyde(MDA) and ferritin. Correlation of MDA plasma concentrations and serum ferritin concentrations in MDS patients and healthy donors. Values of ferritin are in decadic logarithm and regression is logarithmic. *P* and *r* values were derived by a *Pearson* correlation test.

4. Discussion

Our results revealed that plasma concentrations of total, oxidized, and reduced forms of Cys, Hcys, and CG were significantly elevated in MDS patients versus healthy donors; conversely, plasma levels of total and oxidized GSH and nitrite were significantly depressed in MDS patients compared to the control group. Moreover, significant concentration differences of nitrite, t-CG, and ox-CG were found between the clinical subgroups of MDS patients and the controls. We also found that patients requiring chelation therapy and those receiving transfusions had significantly higher levels of both MDA and free iron, whereas aminothiols, nitrite, and nitrate compounds did not differ between these groups and the compared patient groups not requiring chelation treatment or the nontransfused patients. The question arises of what is the cause and significance of these newly elucidated findings.

We found significantly depressed levels of nitrite in all groups of MDS patients versus controls. These results were in accordance with our previous study of middle age patients with MDS [16]. However, the age of the subjects in our study group, was in the range 25–91 years. The relation of plasma levels of nitric oxide to the patient age depends on the substances (NO_2^-, NO_3^-) measured to assess NO [17–19]. Alusik et al. found that nitrite levels in elderly patients (over eighty) were slightly, nonsignificantly lower than in a younger control group in their thirties, whereas nitrate concentrations were nonsignificantly higher in elderly patients than in these controls [20]. Mikiwa et al. found that together, both males and females showed a nonsignificant enhancement of nitrite and nitrate with age [21]. Even the levels of aminothiols are believed to vary with age. Bates et al. found in a study of young people aged 4–18 years, compared with people aged 65 years and over, that both Hcys and Cys exhibited progressive increases with age throughout the age range whereas CG plasma concentration did not change significantly with age [22]. According to our previous

discussions a correct assessment of the importance of nitric oxide and CG levels estimated in our MDS patients should not be influenced by age, because these relations were referred to as non-significant, as shown above. Collecting a control group of healthy individuals of older age is rather problematic, due to other diseases typical for the elderly population (hypertension, diabetes, etc.). Optimally, the control group should match for as many parameters as possible. As MDS usually occurs in elderly patients, the control group should be of similar age range. However, this matching may face other limitations—the primary difficulty is to find healthy individuals of older age who do not suffer from other abovementioned diseases which may significantly affect the obtained results. Further, MDS is not limited to elderly patients only. Considering that, we decided to compare our patient group with a healthy control group of lower age range to possibly observe all the changes that would occur.

Association between decreased nitrite concentration and oxidative stress (increased levels of MDA) in healthy humans has been described [23]. Modun et al. found significantly decreased levels of plasma nitrite and significantly enhanced levels of MDA in healthy donors after hyperoxia. The authors supposed the role of the generated ROS and their rapid reaction with NO in generating peroxynitrite and hence decrease in NO bioavailability. They also considered diminish production of NO by endothelial NO synthase (eNOS) due to the decrease of cofactor of eNOS, tetrahydrobiopterin, through its oxidation or eNOS uncoupling in the presence of ROS and peroxynitrite. In our study the significantly lowered levels of nitrite in the studied group of MDS patients versus the controls could be induced by a combination of more than one factor. One consideration is the possible consumption of NO by an elevation of plasma free hemoglobin levels [24]. Thus, we further sorted the patients into transfused and nontransfused groups. Consequently, we tested the hypothesis that transfusion lowered patient nitrite levels, as a consequence of NO consumption by free hemoglobin. In the samples of patients who received transfusions, we did not find significantly lowered levels of nitrite compared to patients without transfusions ($P = 0.137$; two-tailed, two-sample Student's t-test), although they tended to be lower. We found significantly enhanced levels of MDA in transfused patients. Moreover, nitrite concentrations moderately negatively correlated with MDA levels ($r = -0.339$, $P = 0.006$) (Figure 7), using a Spearman's rank correlation test. It has been described that free hemoglobin in plasma may increase MDA concentrations in plasma [25] and hence could contribute to oxidative stress in MDS patients. Enhanced oxidative stress may oxidize tetrahydrobiopterin, the crucial cofactor of eNOS, and its deficiency may result in eNOS uncoupling [26]. Furthermore, eNOS could be inhibited by enhanced levels of asymmetric dimethylarginine which has been described in plasma of MDS patients [16]. Our study does not reveal the underlying mechanism of depressed concentration in nitrite in plasma of MDS patients. We suppose that oxidative stress in MDS patients could contribute to disruption of eNOS. However, differences in age, diet, and consumption by free hemoglobin should be also considered. This problem remains to be elucidated in further studies.

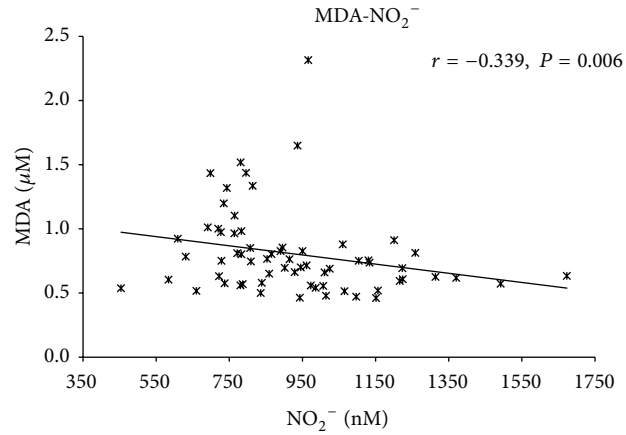

Figure 7: Correlation of total malondialdehyde (MDA) and nitrite (NO_2^-). Correlation of MDA plasma concentrations and nitrite plasma concentrations in MDS patients and healthy donors. P and r values were derived by a Spearman's rank correlation test.

A negative correlation of plasma nitrite with all forms of CG was found in our study. We also found significantly higher plasma levels of CG together with Cys and Hcys. Aminothiols, especially Hcys, are a common cardiovascular risk factor. In our study there were 19 patients with cardiovascular event and 28 patients with hypertension. We tested hypotheses that patients with cardiovascular event or hypertension had higher levels of Cys, CG, and Hcys. Using two-tailed, two-sample Student's t-test we have found significant differences neither in Hcys levels nor in levels of CG among groups of patients with cardiovascular event and hypertension comparing to group of patients without mentioned comorbidities. Only levels of total Cys were significantly higher in patients with hypertension ($P < 0.03$). The higher levels of total Hcys in MDS have been described earlier and were in accordance with Cortelezzi et al. [6]. Elevated levels of Cys had not yet been described in relation with MDS. De Chiara et al. studied Cys in cardiovascular disease, and they have suggested that Cys is the main plasma antioxidant compound, with its concentration reflecting increased oxidative processes [27]. However, an association has been described between Cys and older age [28]. As mentioned, CG was described to have a lack in correlation with age [22]. This dipeptide has not been as widely studied as GSH and Hcys; however, its role in the pathophysiology of several diseases has been described previously [29]. We found that levels of all forms of CG were significantly higher in MDS patients, and moreover, levels of t-CG differed significantly between each of the MDS clinical subgroups and levels of ox-CG were significantly higher in subgroup 1 of MDS compared to subgroup 4 and healthy donors. CG together with Cys is the main components of GSH metabolism and essential substrates for GSH synthesis.

Reduced levels of plasma GSH as a consequence of oxidative stress have been described in several works [30–32]. Intracellular GSH is a key antioxidant involved in the protection of the cell against oxidative radicals forming GSH disulfide and in the metabolism of endogenous and

xenobiotic compounds to yield mostly thioethers. GSH has also been described to play a critical role in determining apoptosis sensitivity and resistance in leukemia cells [33, 34]. Once GSH is oxidized, it is exported out of the cell and degraded [35]. GSH is catabolized through the action of GGT to γ-glutamyl moiety, coupled to another amino acid and CG, which can be further catabolised to Cys and glycine. Significantly reduced levels of total and oxidized GSH, together with enhanced levels of Cys and CG, suggest a possible imbalance in GSH metabolism in MDS. In our study, the activity of GGT, a crucial enzyme in the metabolism of GSH in MDS patients, was 0.60 μkat/L, which is mostly at the upper limit of the standard reference interval of GGT (0.16–0.8 μkat/L). Moreover, levels of GGT were moderately negatively correlated with total GSH concentrations. GGT has been described as a marker of oxidative stress [31–33], and several studies support the view that the enhanced expression of GGT may represent an important factor in the development of a more aggressive and resistant phenotype of cancer cells [36–38]. In addition, De Donatis et al. showed that the blocking of GSH metabolism through GGT inhibition elicited an extralenticular accumulation of GSH and the ability of CG to abolish this effect [39]. In our study, the significantly elevated levels of CG as a consequence of enhanced GGT activity could be considered.

Ghoti et al. described lower levels of reduced GSH in the red blood cells, platelets, and neutrophils of MDS patients with RARS and RCMD. These data correlated with serum ferritin levels and were attributed to oxidative stress due to iron overload [4]. In our study, MDS patients had significantly depressed plasma levels of t-GSH and ox-GSH as compared with healthy donors. In several works, oxidative stress in MDS was attributed to the early stages of MDS, characterized by enhanced apoptosis and transfusion therapy [2, 40]. These patients suffer from iron overload and consequently oxidative stress development [41]. We found enhanced plasma levels of aminothiols (Cys, CG, and Hcys) in groups 1 and 2, respectively. These groups involve MDS subtypes designated as early stages of MDS. However, serum ferritin levels and serum free iron did not differ significantly between each of the MDS subgroups (1–4) in our study. Meanwhile, the oxidative stress represented by MDA concentrations was significantly higher in patients with iron overloads (meaning patients requiring chelation therapy and transfused patients). MDA had a moderate correlation with iron and ferritin levels. However, other analyzed compounds lacked correlation with oxidative stress as a consequence of iron overload. This conflict with Ghoti et al. may be explained by experimental conditions. We evaluated levels of GSH in plasma and Ghoti et al. did so in cells. We suggest possibility that there is an oxidative stress as a result of iron overload; however, the imbalance in plasma aminothiols, nitrite, and nitrate compound could be probably influenced by other factors.

5. Conclusions

In conclusion, we simultaneously determined nitrite, and nitrate; plasma aminothiols, and MDA in 61 MDS patients in the context of clinical outcomes and as a consequence of iron overload and compared both with 23 healthy donors. Our results revealed for the first time the significant differences in the concentrations of total plasma aminothiols in MDS patients and no correlation of these parameters with iron overload represented by serum iron/ferritin levels. We suggest that oxidative stress could participate in the development of MDS disease, not only to be consequence of iron overload. This work brings about new insight into the problematic nature of MDS and oxidative stress. However, further studies are needed to clarify this subject more concretely.

Conflict of Interests

Any significant amount of data reported in our paper has not been published elsewhere, nor is it under consideration for publication elsewhere. The authors certify that there is no conflict of interests, and that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the paper.

Authors' Contribution

Kristýna Pimková performed HPLC analysis of nitrite and MDA and wrote the paper, Jiří Suttnar carried out data analysis and interpretation and wrote the paper, Leona Chrastinová performed analysis of aminothiols, Roman Kotlín performed analysis of nitrate, Jaroslav Čermák provided clinical data and patient samples, and Jan Evangelista Dyr conceived the study and wrote the paper.

Acknowledgments

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