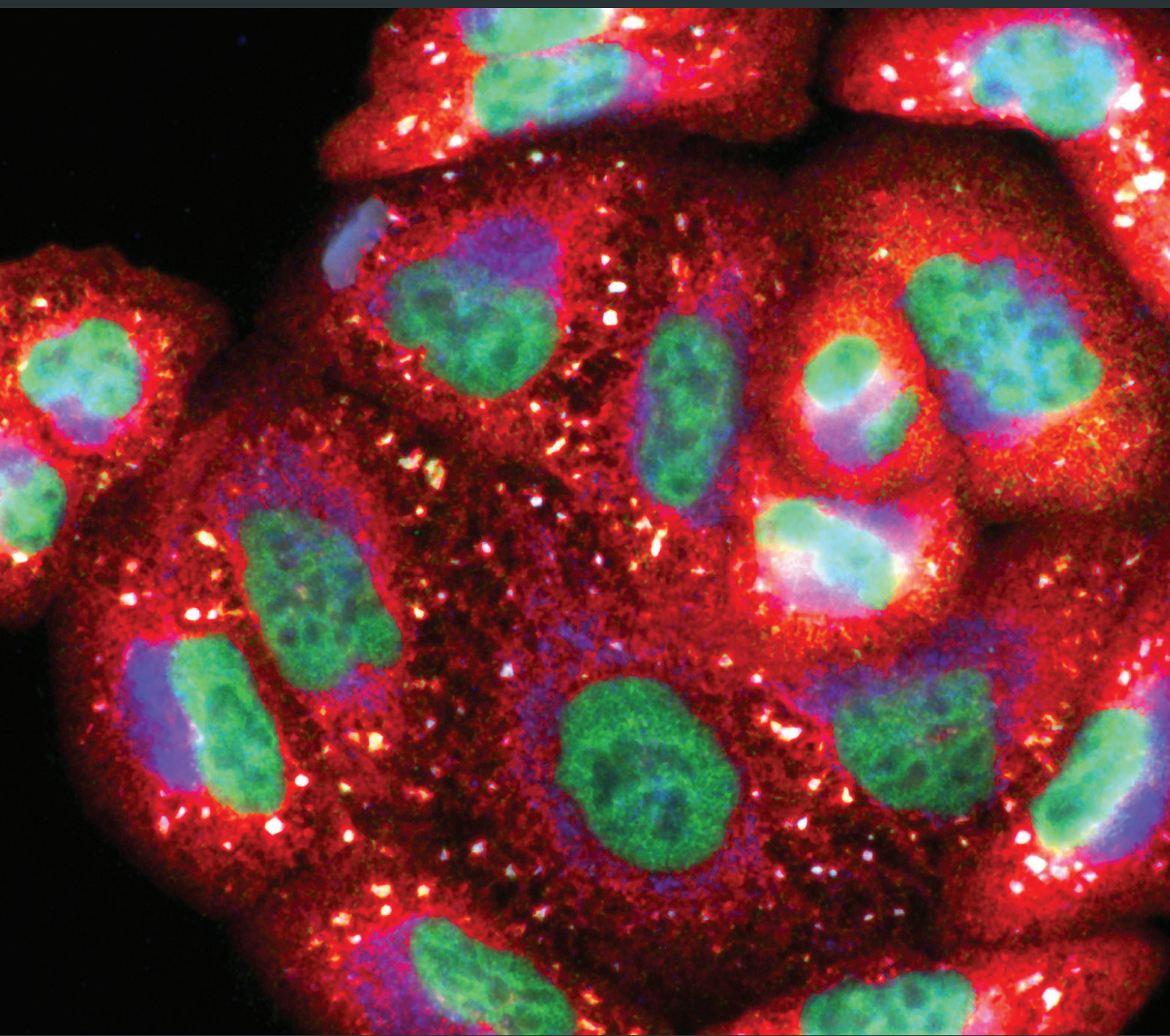
Oxidative Medicine and Cellular Longevity

Lipid Peroxidation Products in Human Health and Disease 2016

Guest Editors: Kota V. Ramana, Sanjay Srivastava, and Sharad S. Singhal



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Editorial Board

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Editorial

Lipid Peroxidation Products in Human Health and Disease 2016

Kota V. Ramana,[1] Sanjay Srivastava,[2] and Sharad S. Singhal[3]

[1]*Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555, USA*
[2]*Environmental Cardiology, University of Louisville, Louisville, KY 40202, USA*
[3]*Department of Molecular Medicine, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA 91010, USA*

Correspondence should be addressed to Kota V. Ramana; kvramana@utmb.edu

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Lipid peroxidation has been implicated in the etiology of several diseases. The process of lipid peroxidation can be initiated by a variety of oxidants, including H_2O_2, superoxide, and the highly reactive hydroxyl radicals during pathological conditions or exposure to xenobiotics and environmental pollutants. Lipid peroxidation can alter vital membrane protein structure and function, and if unchecked, it could lead to cellular dysfunction and widespread tissue damage. Despite multiple studies showing that uncontrolled and excessive production of lipid peroxidation products during oxidative stress are the main cause of various disease complications, the mechanisms by which lipid peroxidation products regulate oxidative, immune, and inflammatory responses remain unclear. Therefore, understanding the role of various lipid peroxidation products in mediation of oxidative and inflammatory signaling is potentially important in developing better therapeutic strategies. In the series of special issues, we are continuously highlighting the significant role of lipid peroxidation products in human health and disease.

The 3 review articles published in this issue discussed how oxidative stress and lipid peroxidation products are involved in various pathological conditions. An excellent and informative review article by S. Q. Rodríguez-Lara et al. described the relationship between oxidative stress and ischemia/reperfusion (I/R) lesions. Specifically, they discussed how current pharmaceutical and mechanical interventions for I/R, although promising, cannot be used in all patients. Further, the significance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the I/R has been exclusively discussed and suggested possible antioxidative stress therapeutic regimens in the treatment of I/R lesions. Another review article by J. Schroter and J. Schiller described the significance of chlorinated phospholipids as biomarkers of oxidative stress in various inflammatory diseases. Specifically, authors have nicely discussed the formation of chlorinated phospholipids, interaction with cellular biomolecules, and their involvement in various pathological conditions including atherosclerosis and arthritis. Most importantly, authors have discussed various mass spectrometry and chromatographic and immunological methods for the analysis of chlorinated phospholipids. Z. Qiao et al. in their review article discussed how dysfunctional autophagy is involved in the disease vitiligo by altering the redox homeostasis in the melanocytes. In this review, authors carefully discussed how oxidative stress and lipid oxidation could be involved in the pathophysiology of vitiligo. Further, they discussed how autophagy is regulated by the oxidative stress and its involvement in the melanocyte destruction leading to the vitiligo complication. The review articles in this special issue provide widespread information on the how oxidative stress and lipid aldehydes are involved in the human health and diseases.

The research article by M. Galicia-Moreno et al. investigates the role of oxidative stress markers in alcoholic liver cirrhosis patients. In this cross-sectional study comprising of 187 Latin-American patients, in addition to liver function tests, authors have also analyzed various oxidative stress markers in the blood such as lipid peroxidation, carbonylated protein and glutathione (GSH), and oxidized glutathione (GSSG). Interestingly they found that serum malondialdehyde, a marker for lipid peroxidation, levels are increased in proportion to the severity of the liver damage. At the same time, the levels of GSH and GSSG are varied according to the

different stages of the liver cirrhosis. This study indicates that the increase in the oxidative stress markers was seen at the early stages of disease severity in alcoholic cirrhotic patients and abstinence from alcohol consumption restores GSH in patients with advanced disease severity.

Another cross-sectional study by S. Carrillo-Ibarra et al. reported the oxidative stress and inflammation status in patients with acute graft dysfunction (AGD) with Tacrolimus. In this study, authors have examined various oxidative stress markers such as lipid peroxidation and superoxide dismutase (SOD) and inflammatory markers such as TNF-alpha, IL-6, C-reactive protein, and nitric oxide in the serum. The results indicate that in patients with AGD there was an increase in the lipid peroxidation and levels of 8-isoprostanes and decrease in nitric oxide and SOD. However, no significance differences between serum TNF-alpha, IL-6, and C-reactive protein were observed in patients with or without AGD. These results suggest that deregulation of oxidative stress but not inflammation may be responsible for AGD.

A research study by H. Sonowal et al. examined the effect of aspalatone in preventing the endothelial dysfunction using cultured human aortic endothelial cells. Their results indicate that aspalatone prevents VEGF-induced lipid peroxidation in endothelial cells. Further, they have shown that aspalatone prevents VEGF-induced endothelial dysfunction as determined by examining the levels of eNOS, iNOS, ICAM-1, and VCAM-1. They have also shown that aspalatone prevents the VEGF-induced inflammatory response in endothelial cells. These results suggest a novel use of aspalatone in complications related to endothelial dysfunction.

In conclusion, we believe that our series of special issues on this research topic will continue to highlight the significance of lipid peroxidation products in human health and disease. A number of recent studies suggest that oxidative stress-generated lipid peroxidation products, which regulate cellular signaling pathways play a critical role in the pathophysiology of a number of disease complications. Several oxidative stress markers are now recognized as biomarkers of many human diseases and therapeutical approaches that potentially alter oxidative stress and generation of lipid peroxidation products are shown to be effective in controlling various human diseases.

Acknowledgments

We would like to thank all the authors and reviewers who took part in the success of this special issue. We also acknowledge great support of editorial staff of the Journal.

Kota V. Ramana
Sanjay Srivastava
Sharad S. Singhal

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Research Article

Aspalatone Prevents VEGF-Induced Lipid Peroxidation, Migration, Tube Formation, and Dysfunction of Human Aortic Endothelial Cells

Himangshu Sonowal, Pabitra B. Pal, Kirtikar Shukla, and Kota V. Ramana

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555, USA

Correspondence should be addressed to Kota V. Ramana; kvramana@utmb.edu

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Although aspalatone (acetylsalicylic acid maltol ester) is recognized as an antithrombotic agent with antioxidative and antiplatelet potential; its efficacy in preventing endothelial dysfunction is not known. In this study, we examined the antiangiogenic, antioxidative, and anti-inflammatory effect of aspalatone in human aortic endothelial cells (HAECs). Specifically, the effect of aspalatone on VEGF-induced HAECs growth, migration, tube formation, and levels of lipid peroxidation-derived malondialdehyde (MDA) was examined. Our results indicate that the treatment of HAECs with aspalatone decreased VEGF-induced cell migration, tube formation, and levels of MDA. Aspalatone also inhibited VEGF-induced decrease in the expression of eNOS and increase in the expression of iNOS, ICAM-1, and VCAM-1. Aspalatone also prevented the VEGF-induced adhesion of monocytes to endothelial cells. Furthermore, aspalatone also prevented VEGF-induced release of inflammatory markers such as Angiopoietin-2, Leptin, EGF, G-CSF, HB-EGF, and HGF in HAECs. Thus, our results suggest that aspalatone could be used to prevent endothelial dysfunction, an important process in the pathophysiology of cardiovascular diseases.

1. Introduction

Endothelial cells that form new blood vessels play an important role in the pathophysiology of cardiovascular diseases (CVD) [1]. Although, normal endothelial cell function is involved in various physiological processes such as angiogenesis, embryonic development, wound healing, and tissue remodeling, abnormal endothelial function could lead to increased vascular complications, cancer growth, and metastasis [2–4]. Factors that interrupt normal endothelial cell functions such as injury, lipid infiltrations, oxidative stress, and inflammatory response could lead to endothelial dysfunction [5, 6]. Several studies have also shown that the widespread vascular endothelial activation, dysfunction, and damage lead to multiple organ failure during severe bacterial infections and in sepsis [7]. Furthermore, endothelial function that leads to angiogenesis is involved in the tumor development, survival, invasion, and metastasis [3, 8]. Several studies have shown the significance of vascular endothelium in various disease pathologies and identified several agents that can prevent endothelial dysfunction as potential agents to control CVD [2, 4, 6, 9].

During pathological conditions, increased oxidative stress generates reactive oxygen species (ROS) via NAPDH oxidase/mitochondrial pathways. Increased ROS formation leads to peroxidation of lipids and release toxic lipid aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal. The lipid aldehydes could act as secondary signaling intermediates to alter signaling pathways responsible for cellular equilibrium leading to change in the endothelial cell phenotype, increase proinflammatory gene expression, tissue damage, and endothelial dysfunction [10–13]. Increased expression of FGF and VEGF during oxidative stress induces endothelial dysfunction [14–17]. Besides CVD, VEGF has also been shown to be involved in endothelial dysfunction in patients with preeclampsia and chronic kidney disease [18, 19]. Several VEGF inhibitors have been synthesized and tested as potential CVD drugs. However, some of the VEGF inhibitors have been shown to cause side effects such as increased endothelial toxicity, predisposing to thrombosis

and hypertension [20–23]. Besides several synthetic anti-VEGF agents, several reports also suggest the significant use of various antioxidants in the prevention of endothelial dysfunction [24, 25]. Antioxidants such as curcumin, quercetin, resveratrol, and N-acetylcysteine have been shown to prevent endothelial dysfunction in preclinical studies [24, 26–29]. Although, some of these antioxidants have gone to clinical trials for the treatment of CVD, they are not as effective as they are in experimental animals [30, 31]. Therefore, alternative antioxidant regimens are required to control endothelial dysfunction.

Aspirin (acetylsalicylic acid) is a frontline antipyretic and anti-inflammatory agent with potent antithrombotic activity, saving millions of lives in patients with CVD [32, 33]. Besides CVD, aspirin has also been shown to decrease the rate of cancer growth and metastasis and improved overall survival rate in patients [34–36]. Furthermore, aspirin has been shown to be effective in inhibiting the release of various inflammatory cytokines responsible for CVD [37, 38]. However, side effects such as a gastrointestinal insult have been reported with higher doses of aspirin use [39]. Even with short term administration, aspirin reportedly exhibited both gastric and duodenal mucosal damage [40]. Thus, to overcome these problems, different formulations of antithrombotic agents with similar therapeutic potential but lesser side effects have been developed.

Aspalatone is a potent antioxidant synthesized by the esterification of acetylsalicylic acid (aspirin) and maltol [41–43]. Compared to aspirin, aspalatone has been reported to show negligible gastrointestinal damage and possesses potent antithrombotic activity [41, 44, 45]. Aspalatone also inhibits collagen-induced platelet aggregation in vitro and in vivo (IC_{50} = 180 μM) [45]. However, the effect of aspalatone in preventing endothelial dysfunction is not known. In the present study, we examined the effect of aspalatone on VEGF-induced endothelial cell growth, migration, tube formation, and monocyte adhesion. Our results indicate that aspalatone prevents VEGF-induced endothelial cell dysfunction by preventing VEGF-induced increase in the lipid peroxidation, inflammatory and adhesion maker expression, and iNOS and VCAM-1 expression and decrease in eNOS levels. Thus, our results suggest that aspalatone, a novel derivative of aspirin, could be used for the prevention of endothelial dysfunction and its associated complications.

2. Material and Methods

2.1. Materials. Aspalatone was purchased from Cayman Chemical Company. Endothelial Cell Medium (ECM) was obtained from ScienCell™ Research Laboratories. PBS, penicillin/streptomycin solution, and Trypsin/EDTA were obtained from Invitrogen. FBS was obtained from Gemini Bio-Products. MTT was obtained from Sigma. RIPA buffer was obtained from Santa Cruz Biotechnology. Vascular Endothelial Growth Factor (VEGF-165) and antibodies against VCAM-1, ICAM-1, eNOS, and GAPDH were obtained from Cell Signaling Technologies. Antibody against iNOS was obtained from Abcam. CM-H_2DCFDA was obtained from Molecular Probes, Invitrogen. Malondialdehyde (MDA) detection kit was obtained from Oxis Research. Human Milliplex angiogenic cytokine multiplex kit was obtained from Millipore. All other reagents and chemicals used were of analytical grade and were obtained from Sigma.

2.2. Cell Culture. Primary human aortic endothelial cells (HAECs) were obtained from ScienCell™ Research Laboratories and were grown in complete Endothelial Cell Medium (ECM) supplemented with endothelial growth factors along with 5% FBS and 1% penicillin/streptomycin. All the cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

2.3. Measurement of Cytotoxicity. HAECs (3000 cells/well) were growth-arrested with 0.1% serum containing different concentrations of aspalatone (25–200 μM) without or with VEGF (10 ng/mL). After 24 h, cell viability was determined by MTT assay.

2.4. Cell Migration Assay. HAECs were seeded in 12-well plates and allowed to form a confluent monolayer. The cells were then growth-arrested overnight in 0.1% FBS containing media. A longitudinal uniform scratch at the center of the monolayer was made with a 10 μL sterile pipette tip carefully, and the monolayer was washed 3x with serum-free media. HAEC media with 0.1% FBS and VEGF (10 ng/mL) containing vehicle or aspalatone (50 μM) was added to the wells. The wells were photographed at 0 h and 18 h. Percentage changes in the cell migration were calculated by formula $(\text{Width}_{0\,\text{hr}} - \text{Width}_{18\,\text{hr}})/\text{Width}_{0\,\text{hr}} \times 100$.

2.5. In Vitro Capillary Tube Formation Assay. HAEC tube formation assay was performed by using an in vitro angiogenesis kit from EMD Millipore following manufacturer's instructions. Briefly, 50 μL of diluted ECM matrix solution was added to each well of 96-well plate and incubated at 37°C to allow the matrix to solidify. Growth arrested HAECs were harvested and then seeded carefully without disturbing the solidified matrix at 1×10^4 cells/well in the absence and presence of aspalatone (10 μM, 20 μM, 50 μM, and 100 μM) and incubated for additional 18 h. Photographs were taken and the length of the capillary network was quantitated as described earlier [46, 47].

2.6. Monocyte-Adhesion Assay. In vitro monocyte cell adhesion assay was performed as described earlier [46]. Briefly, HAECs were seeded in 96-well plates at a density of 3000 cells/well and subconfluent cells were treated with aspalatone (50 μM) overnight. After incubation, the cells were rinsed with serum-free media and THP-1 cells were added in a ratio of 1 : 3 (HAEC : THP-1) in the absence or presence of aspalatone and VEGF (10 ng/mL) for another 18 h. At the end of the incubation period wells were washed and 100 μL of media containing MTT was added and incubated for another 3 h. Formazan crystals were dissolved by the addition of DMSO and absorbance was recorded at 570 nm using a plate reader.

2.7. Measurement of ROS Accumulation in HAECs. Intracellular ROS accumulation was measured by flow cytometry using CM-H_2DCFDA. CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA used for measuring reactive oxygen species (ROS) in cells. To analyze ROS levels, the cells were treated for 18 h with VEGF (10 ng/mL) in the absence or presence of aspalatone (50 μM). After the incubation period, the cells were stained with CM-H_2DCFDA for 5 min, harvested, and analyzed immediately by a Flow Cytometer (BD LSRII Fortessa). Data analysis was performed using Flow Jo (Treestar, Ashland, OR, USA) and represented as fold change of Mean Fluorescence Intensity (MFI) compared to control.

2.8. Analysis of MDA Levels in HAECs. Lipid peroxidation marker, MDA, levels were measured in HAECs by using a kit from Oxis International Inc., following manufacturer's instructions. Briefly, the cells were treated for 18 h with VEGF (10 ng/mL) in the absence or presence of aspalatone (50 μM). The cells were then washed with PBS 2x and lysed in PBS by sonication. Cell debris was removed by centrifugation at 3000 ×g for 10 minutes at 4°C. The supernatant was used in the assay as per the instructions and the absorbance was recorded at 586 nm using a plate reader. Total MDA levels (μM) were calculated based on the standard curve and normalized to protein levels.

2.9. Analysis of Inflammatory Cytokines Secreted by HAECs. Analysis of various cytokines secreted by HAECs treated with VEGF (10 ng/mL) in absence or presence of aspalatone (50 μM and 100 μM) was analyzed by using a Human Angiogenesis/Growth Factor Magnetic bead panel kit from Millipore following manufacturer's instructions. Briefly, the culture media was collected, concentrated by lyophilization, and incubated with the labelled magnetic beads. After overnight incubation, the beads were counterstained with Streptavidin-Phycoerythrin and analyzed with a Luminex™ analyzer from Millipore. The results are expressed as pg/mL based on the standard curve generated with the standards.

2.10. Western Blot Analysis. Equal amounts of cell lysates (40 μg) were loaded and separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 5% nonfat dried milk and incubated with the specific primary antibodies at 4°C overnight followed by incubating with the specific secondary antibodies. Immunolabeling was detected using SuperSignal™ West Pico Chemiluminescent Substrate (ECL) from Thermo Scientific following manufacturer's instructions. The membranes were stripped with Restore™ PLUS stripping buffer from Thermo Scientific following manufacturer's instruction for reprobing with other antibodies or GAPDH.

2.11. Statistical Analysis. Data presented as mean ± SD and p values were determined by using an unpaired Student's t-test from Microsoft Office Excel 2007 software. $p < 0.01$ was considered as statistically significant.

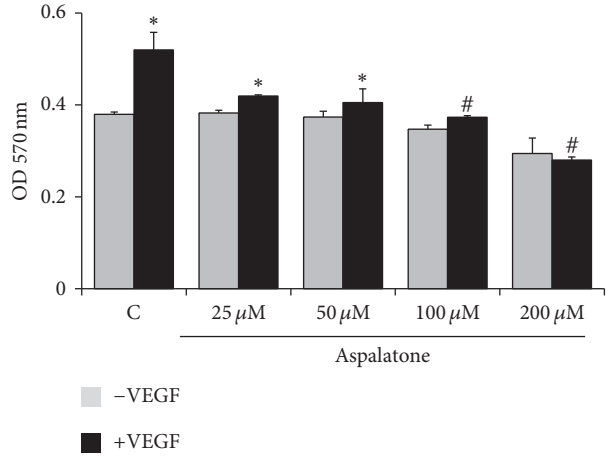

Figure 1: Effect of aspalatone on HAECs viability: HAECs (3000 cells/well) were treated with indicated concentrations of aspalatone without or with VEGF (10 ng/mL). After 24 h, cell viability was determined by MTT assay. Values are mean ± SD ($n = 5$). $^{*}p < 0.05$ when compared with control and $^{\#}p < 0.005$ when compared with VEGF- treated group.

3. Results

3.1. Effect of Aspalatone on HAECs Viability. The effect of aspalatone on VEGF-induced cell viability is not known; therefore, we first examined the effect of aspalatone on viability of HAECs. As shown in Figure 1, treatment of cells with aspalatone alone did not cause any significant increase in the cell growth or death when measured using aspalatone concentrations at 25, 50, 100, and 200 μM. However, treatment of HAECs with VEGF increased cell growth and this increase was prevented by pretreatment of aspalatone. Thus, our results suggest that aspalatone alone did not show any significant effect on HAEC viability but decreased VEGF-induced increase in the cell growth.

3.2. Effect of Aspalatone on VEGF- Induced HAECs Migration and Tube Formation. We next examined the efficacy of aspalatone in the prevention of VEGF-induced cell migration. In vitro wound healing assay was performed in cells treated with VEGF in the absence and presence of aspalatone. The results shown in Figures 2(a) and 2(b) indicate that VEGF caused migration of HAECs, which is observed by complete closure of the wound; however, pretreatment of aspalatone prevented the VEGF-induced migration of HAECs. Since migration of cells is an important step for neovascularization, we next examined the effect of aspalatone on VEGF-induced tube formation in vitro, a standard method for determining the angiogenesis in vitro. HAECs were seeded on 96-well plates coated with ECM matrix gel solution. The ECM matrix contained a mixture of growth factors, including VEGF for maximal induction of endothelial cell tube formation. As shown in Figures 3(a) and 3(b), compared to control cells, aspalatone treated cells show significant reduction in the formation of capillary like structures in a dose-dependent manner. Treatment of cells with 50 μM and

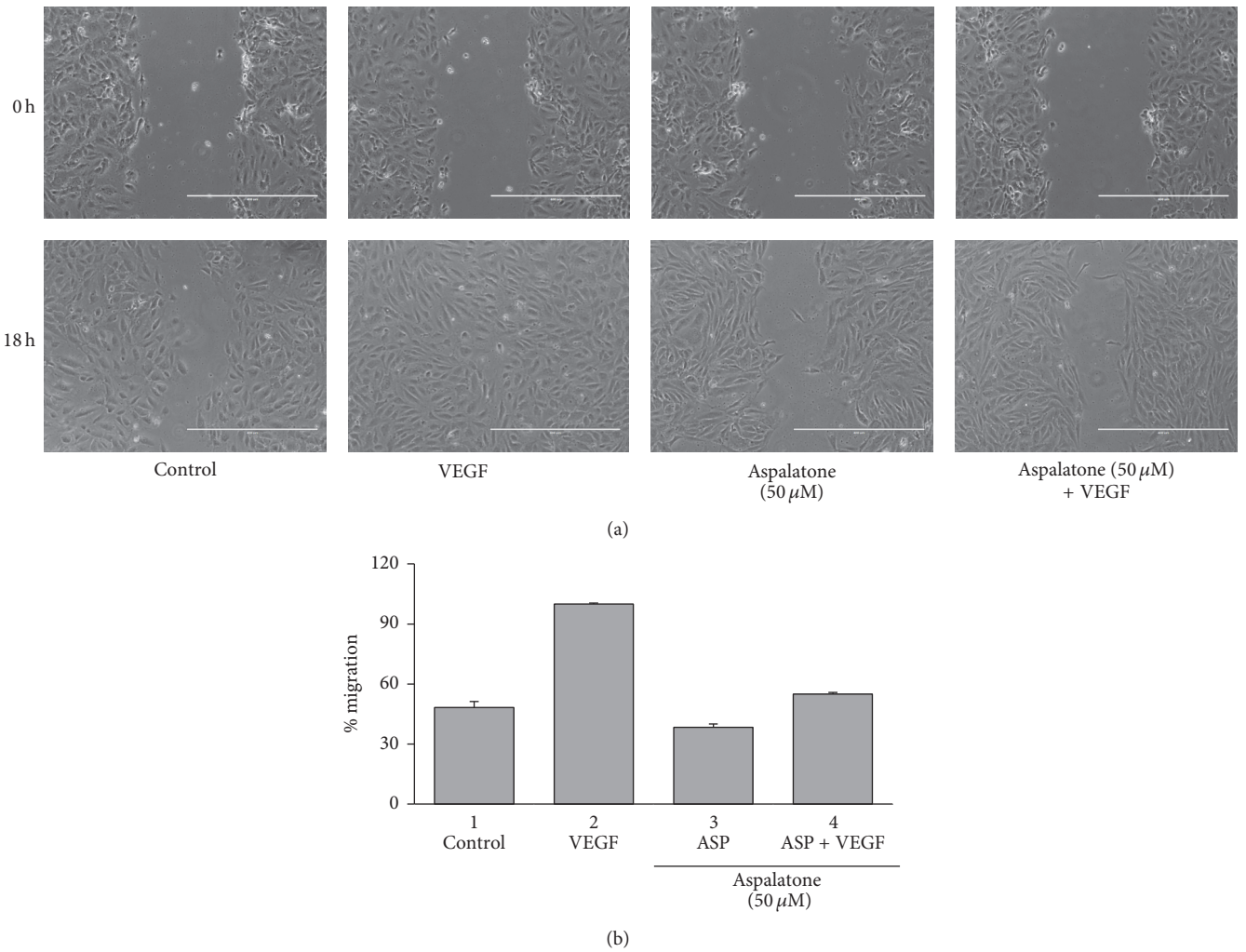

Figure 2: Aspalatone inhibits VEGF-induced migration of HAECs. (a) In vitro wound healing assay showing effect of aspalatone on VEGF-induced migration in HAECs. HAECs were grown in 12-well tissue culture dishes and after the cells became confluent, the monolayer was scratched with a sterile pipette tip. The media was replaced with VEGF (10 ng/mL) without or with aspalatone (50 μM) and the cells were incubated for the indicated time periods. Photographs were taken at 0 h and 18 h when the VEGF containing monolayer wound was completely filled. (b) Histogram showing migration rate calculated by formula $(\text{Width}_{0\,\text{hr}} - \text{Width}_{18\,\text{hr}})/\text{Width}_{0\,\text{hr}} \times 100$. Representative photographs from three independent experiments are shown. Magnification 10x. Scale bar: 400 μm.

100 μM aspalatone significantly prevented the tube formation of endothelial cells, suggesting that aspalatone could prevent neovascularization.

3.3. Effect of Aspalatone on VEGF-Induced Endothelial Dysfunction. Since adhesion of monocytes to the endothelial cells is considered to be one of the initial events in vascular pathologies, we next examined the effect of aspalatone on VEGF-induced monocyte adhesion to HAECs. As shown in Figure 4(a), VEGF caused a significant increase in the adhesion of monocytes to HAECs and in the presence of aspalatone the adhesion of monocytes to HAECs significantly decreased. Since increased expression of adhesion molecules such as ICAM-1 and VCAM-1 has been shown to be responsible for monocyte adhesion, we next examined the effect of aspalatone on VEGF-induced expression of adhesion molecules in HAECs. As shown in Figure 4(b), VEGF alone induced the expression of VCAM-1 and ICAM-1 in HAECs and in cells treated with aspalatone + VEGF, and the expression of VCAM-1 but not ICAM-1 was significantly decreased. However, when compared to controls, aspalatone alone did not increase the expression of these adhesion molecules in HAECs. Thus, these results suggest that aspalatone prevents VEGF-induced monocyte adhesion by preventing the expression of adhesion molecules. We next examined the effect of aspalatone on the expression of eNOS and iNOS in endothelial cells. The data shown in Figure 4(b) suggest that VEGF caused a decrease in the expression of eNOS and increase in the expression of iNOS in the HAECs and aspalatone significantly reversed VEGF-induced changes in the expression of eNOS and iNOS suggesting that aspalatone prevents VEGF-induced endothelial dysfunction.

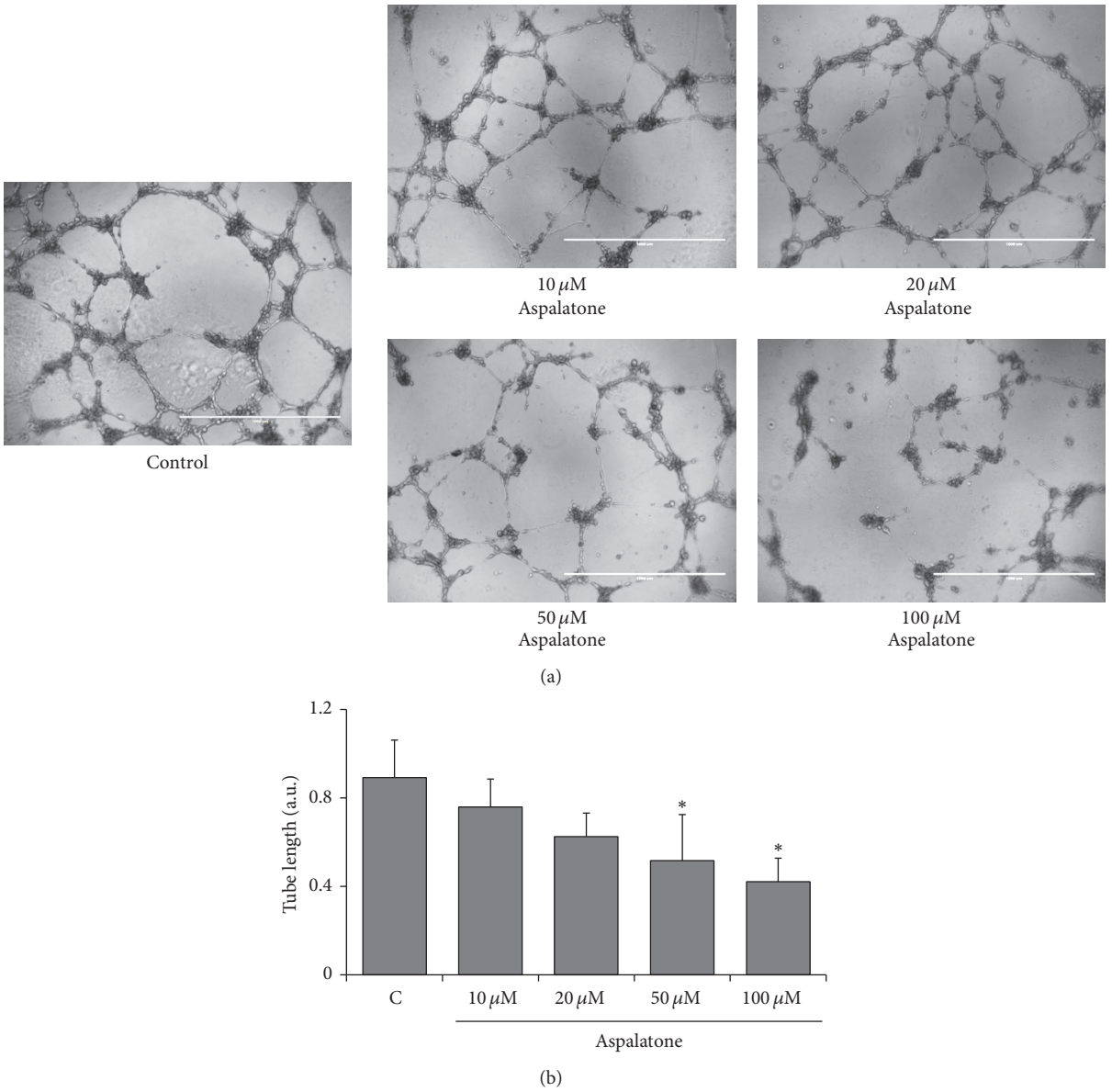

Figure 3: Aspalatone inhibits VEGF-induced tube formation of HAECs. (a) In vitro angiogenesis assay was performed in the absence or presence of different concentrations of aspalatone (10 μM, 20 μM, 50 μM, and 100 μM) by using in vitro angiogenesis assay kit from Millipore. (b) Histograms showing length of capillaries treated with different concentration of aspalatone. Bars represent mean ± SD ($n = 6$). Representative photographs from three independent experiments are shown. Magnification 4x. $^{*}P < 0.05$ when compared with control group. Scale bar: 1000 μm.

To examine if the effect of aspalatone is not only restricted to VEGF but also to other oxidants, we next treated HAECs with lipopolysaccharides (LPS, 1 μg/mL) in the presence and absence of 50 μM aspalatone and examined the expression of eNOS, iNOS, VCAM-1, and ICAM-1. As shown in Figure 4(c), aspalatone also prevented LPS-induced increase in the expression of VCAM-1, ICAM-1, and iNOS in HAECs. Furthermore, aspalatone also restored the LPS-induced decrease in the eNOS levels. Thus, our results suggest that aspalatone could prevent oxidative stress-induced endothelial dysfunction.

3.4. Effect of Aspalatone on VEGF-Induced Expression of Inflammatory Markers. Since it is well known that increased expression of various cytokines and growth factors in HAECs are responsible for endothelial dysfunction, we next examined the effect of aspalatone on VEGF-induced expression of various inflammatory markers in HAECs by using a Millipore multiplex ELISA array. Results shown in Figure 5 indicate a significant increase in the expression of Angiopoietin-2, EGF, Follistatin, Leptin, G-CSF, HB-EGF, HGF, and IL-8 that were observed in the VEGF-treated HAEC culture media, and aspalatone significantly reduced the VEGF-induced release

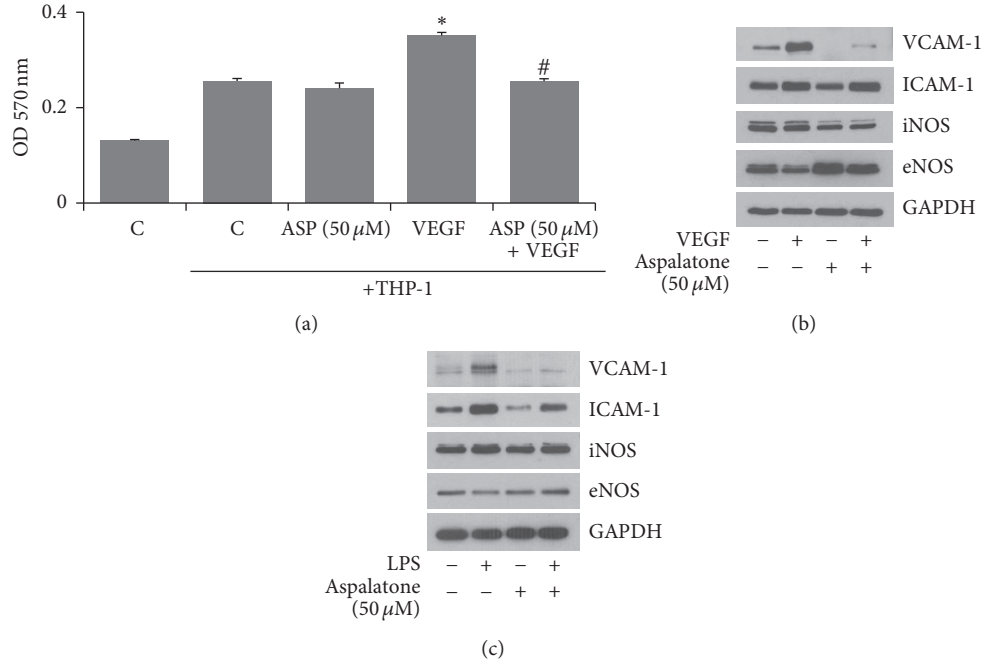

Figure 4: Aspalatone inhibits VEGF-induced adhesion of monocytes to HAECs. (a) HAECs (3000 cells/well) in 96-well plates were treated with aspalatone (50 μM) overnight followed by addition of THP-1 cells and VEGF (10 ng/mL) for another 18 h. Cell adhesion was determined by MTT absorbance recorded at 570 nm using a plate reader. Bars represent mean ± SD ($n = 5$). $^{*}p < 0.05$ when compared with control and $^{\#}p < 0.005$ when compared with VEGF-treated group. HAECs treated without or with (b) VEGF (10 ng/mL) or (c) LPS (1 μg/mL) in the absence and presence of aspalatone (50 μM) for 18 h. Equal amounts of cell extracts were subjected to Western blot analysis using specific antibodies against VCAM-1, ICAM-1, eNOS, iNOS, and GAPDH in HAECs.

of inflammatory cytokines and growth factors in the culture media. These results suggest that aspalatone by preventing the expression of various inflammatory markers prevents VEGF-induced endothelial dysfunction and tube formation.

3.5. Effect of Aspalatone on VEGF-Induced ROS Formation and Lipid Peroxidation. Oxidative stress is the major cause of VEGF-induced endothelial dysfunction and growth factors are well known to exert their effects by increasing the oxidative stress. Therefore, we next examined the effect of aspalatone on VEGF-induced generation of ROS and lipid peroxidation in HAECs. Our results shown in Figures 6(a) and 6(b) indicate an approximately 2-fold increase in the generation of ROS in cells treated with VEGF. This increase in ROS production in HAECs was significantly prevented by aspalatone. Aspalatone alone did not cause any significant changes in the ROS levels and was comparable with the untreated control cells. Since lipid peroxidation is an important event during oxidative stress and acts as an indicator of increased oxidative stress, we next examined the effect of aspalatone on VEGF-induced malondialdehyde (MDA), a marker of lipid peroxidation. The MDA levels in HAECs treated with VEGF in the absence or presence of aspalatone were determined by MDA assay kit. Our results shown in Figure 7 indicate an approximately 1.3-fold increase in the formation of MDA levels in VEGF-treated cells, and this increase was significantly prevented by the aspalatone suggesting that aspalatone prevents VEGF-induced oxidative stress in HAECs.

4. Discussion

Despite extensive evidence that shows the anti-inflammatory, antitumorigenic, and antioxidative activities of aspirin [48–51], to the best of our knowledge, we are not aware of any studies that have documented aspalatone in restoring the growth factor-induced endothelial dysfunction. In this study, we have provided evidence that aspalatone, an esterified derivative of aspirin and maltol, prevents VEGF-induced endothelial dysfunction. Our data suggests that the antioxidant effects of aspalatone prevented the VEGF-induced ROS, lipid peroxidation, and expression of various inflammatory markers in the endothelial cells. Thus, our data for the first time indicate that aspalatone prevents endothelial dysfunction.

Endothelial cells play an important role in atherosclerosis and other cardiovascular complications, including chronic heart diseases [1, 4, 6, 9]. Endothelial dysfunction is associated with an increased risk for CVD [6, 9]. Several studies have shown that oxidative stress-generated ROS levels dictate various cellular processes such as cell growth, differentiation, apoptosis, DNA damage, and tissue injury [52–54]. Antioxidants have attained considerable attention in this regard as various antioxidants have been reported to improve endothelial function by reducing the formation of ROS thereby preserving endothelial cell integrity and

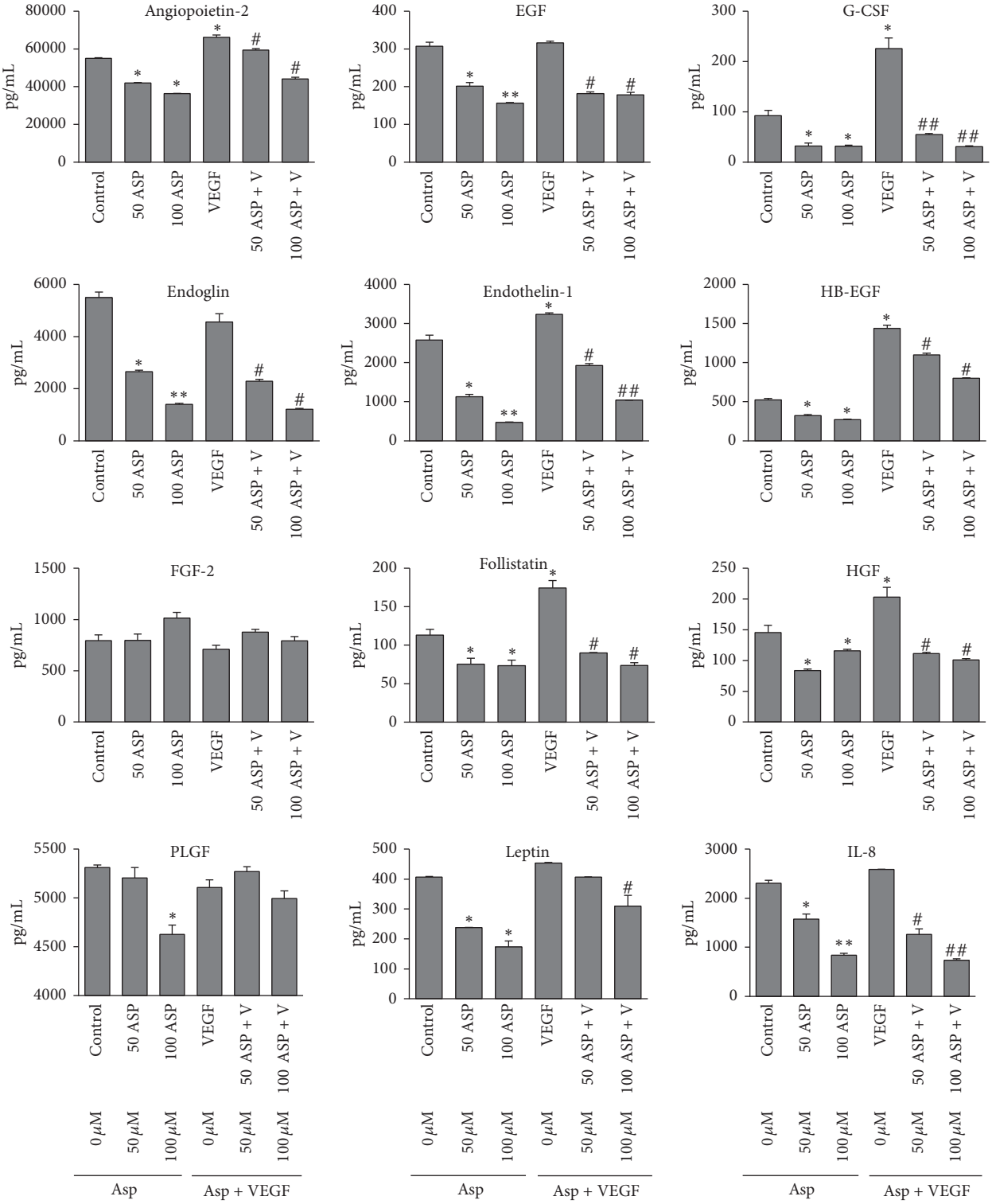

Figure 5: Aspalatone modulates VEGF-induced expression of inflammatory cytokines and growth factors in HAECs. Culture media of HAECs treated with VEGF (10 ng/mL) in the absence or presence of aspalatone (50 μM and 100 μM) were analyzed for inflammatory cytokines by using a Human Angiogenesis/Growth Factor Magnetic bead panel kit from Millipore following manufacturer's instructions using a Milliplex Analyzer System. Bars represent mean ± SD ($n = 4$). $^{*}p < 0.05$ and $^{**}p < 0.001$ when compared with control and $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ when compared with VEGF-treated group.

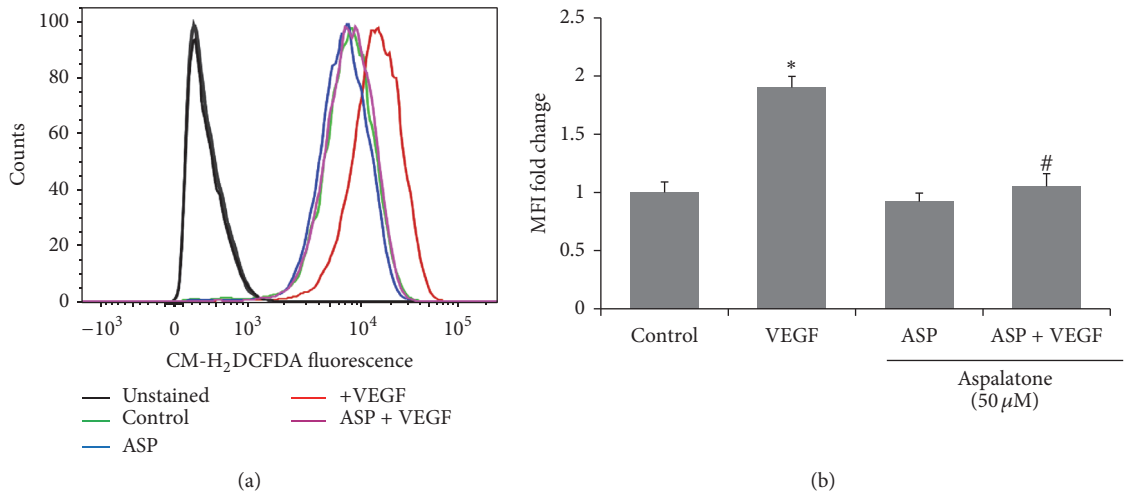

Figure 6: Aspalatone inhibits VEGF-induced reactive oxygen species production in HAECs. (a) Flow cytometric analysis showing CM-H_2DCFDA fluorescence in HAECs treated with VEGF (10 ng/mL) without or with aspalatone (50 μM) for 18 h (red: VEGF alone treated, pink: VEGF + aspalatone, green: control, and blue: aspalatone). Unfilled histograms with solid black lines were unstained controls. (b) Bars showing fold change in CM-H_2DCFDA Mean Fluorescence Intensity (MFI) in HAECs treated with aspalatone (50 μM) in the absence or presence of VEGF (10 ng/mL) for 18 h. Bars represent mean ± SD ($n = 3$). $^*p < 0.05$ when compared with control and $^\#p < 0.001$ when compared with VEGF-treated group.

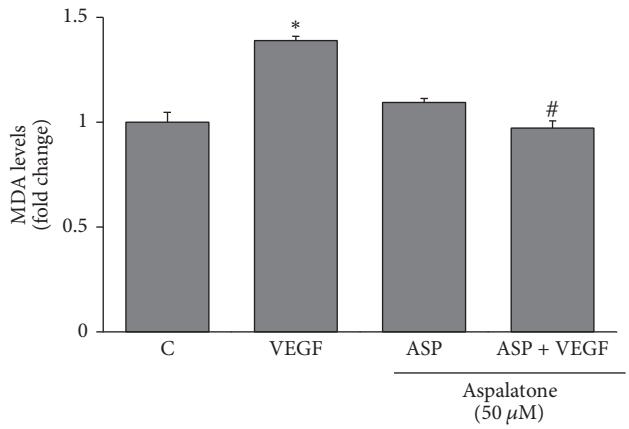

Figure 7: Aspalatone prevents VEGF-induced lipid peroxidation-derived malondialdehyde levels in HAECs. HAECs treated without or with 50 μM aspalatone in the absence and presence of VEGF (10 ng/mL) for 18 h. The MDA levels were measured by using a kit from Oxis International Inc. MDA values (μM) were normalized to total cellular protein content and presented as fold change compared to control. Bars represent mean ± SD ($n = 3$). $^*p < 0.05$ when compared with control and $^\#p < 0.001$ when compared with VEGF-treated group.

function [25–28]. Plant-derived polyphenols have also been reported to improve endothelial cell function and exert a beneficial effect on vascular pathologies [55]. Growth factors such as VEGF bind to its receptors VEGFR1 and VEGFR2 and activate downstream signaling pathways of oxidative stress mediated various kinases such as p38MAPK, AKT, and JNK, which are important regulators of endothelial cell activation [56, 57]. Chiu et al. have shown that β2-glycoprotein-I prevents VEGF-induced HAEC growth as well as migration by regulating the activation of ERK1/2 and AKT [58]. Further, Shu et al. have shown that vasostatin prevents endothelial cell growth by activation of caspase-3 and downregulation of eNOS in HUVEC cells [59]. Similarly, upregulation of Notch signaling by VEGF induces ROS production via activation of Rac1 dependent NADPH oxidase in endothelial cells [57, 60]. ROS in turn activate various redox-sensitive transcription factors such as HIFs, AP-1, and NF-$\kappa\beta$, which transcribe various proinflammatory genes that regulate various signaling pathways leading to endothelial cell activation and dysfunction [61, 62]. Antioxidants such as NAC, Vitamin C, and inhibitors of ROS production have been reported to be effective in preventing endothelial cell activation in culture and animal models [25–29]. In addition, aspirin has been shown to be an antioxidant with anti-inflammatory actions. Aspirin has been shown to reduce the endothelial dysfunction, hypertension, and cardiovascular complications [32, 33]. It is considered as one of the most commonly used analgesic agents that confers significant protection against inflammatory complications, including atherosclerosis, diabetes, and several forms of cancers [32–37]. Our data suggest that aspalatone inhibited VEGF-induced ROS production in HAECs indicating that VEGF-induced endothelial dysfunction may be mediated through ROS. Increased ROS could lead to subsequent reduction in the endothelial Nitric Oxide (NO) availability and decreases eNOS levels. Several studies have shown that decreased eNOS levels could lead to endothelial dysfunction [26, 63, 64]. Since eNOS is a key regulator of vascular tone and antithrombotic process, therefore, we have examined the effect of aspalatone on VEGF- and LPS-induced eNOS. Our results indicate that aspalatone restored the VEGF- and LPS-depleted eNOS levels suggesting that by restoring eNOS levels aspalatone could improve endothelial dysfunction. Consistent with our data,

antioxidants such as curcumin have been shown to restore eNOS levels and improve endothelial dysfunction in vitro and in vivo [24–26].

ROS also induces lipid peroxidation and lipid peroxidation-generated aldehydes are important cellular signaling molecules that regulate various cellular processes [62, 65] including endothelial cells activation and monocyte adhesion [66–68]. Several studies have shown that oxidized lipids and lipid aldehydes such as 4-hydroxynonenal cause endothelial dysfunction leading to monocyte adhesion which play a significant role in vascular inflammation during atherosclerosis [16, 68]. Monocytes have been shown to be important regulators of immune response and play an important role in angiogenesis [69, 70]. Further, several studies have shown that neovascularization is a major contributor to atherosclerotic plaque progression [71]. Our results show that aspalatone prevented VEGF-induced lipid peroxidation-derived aldehyde, malondialdehyde in HAECs, suggesting that aspalatone prevents generation of lipid peroxidation end products responsible for vascular complications. Furthermore, our studies also indicate that aspalatone prevents VEGF-induced monocyte adhesion to endothelial cells by decreasing the VEGF-induced expression of VCAM-1 and ICAM-1. VEGF has been shown to regulate the endothelial function by regulating the expression of ICAM-1 and VCAM-1 that leads to various vascular inflammatory pathologies [72]. Radisavljevic et al. have also shown that VEGF induced endothelial cell migration by upregulating the ICAM-1 expression via PI3K/AKT/NO in brain microvascular endothelial cells [73]. Angiopoietin-1 has been shown to inhibit VEGF-induced leukocyte adhesion to endothelial cells by preventing the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [74]. Various cytokines, chemokines, and growth factors like interleukins (IL-1, IL-6, and IL-8), tumor necrosis factor (TNFs), transforming growth factor beta (TGFβ), granulocyte macrophage-colony stimulating factors (GM-CSF), and interferons have been reported to play important roles in vascular pathologies including atherosclerosis [69, 70, 75]. Various natural antioxidants have been shown to prevent the release of various proinflammatory cytokines and growth factors from endothelial cells [30, 76, 77]. Aspirin, curcumin, resveratrol, and several flavonoid compounds have been shown to control CVD because of their activity in preventing the expression of various inflammatory markers [30–33, 37, 38, 76, 77]. Our results have also demonstrated that aspalatone significantly inhibited the release of various cytokines and growth factors in endothelial cells with or without VEGF treatment. It is possible that aspalatone prevents VEGF-induced endothelial dysfunction by inhibiting the inflammatory cytokines production as well as their autocrine and paracrine crosstalk effects with VEGF.

In summary, although aspirin has also been reported to protect from CVD via its antioxidative and anti-inflammatory properties, some common side effects like gastrointestinal (GI) insult and gastric and duodenal mucosal damage have been reported [39, 40]. Conversely, aspalatone has been shown to have negligible side effects on the GI tract and exhibited similar antioxidant and antiplatelet activity as aspirin [41–45]. However, the effect of aspalatone on endothelial dysfunction is not known. Our studies showed that treatment of HAECs with aspalatone blocked VEGF-induced endothelial dysfunction by restoring the eNOS levels and inhibiting the expression of inflammatory and adhesion molecules. The mechanism of the inhibitory effect of aspalatone on VEGF-induced endothelial dysfunction may involve the antioxidant and anti-inflammatory functions of aspalatone and warrant further investigations in the therapeutic development of this compound.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Alternative Interventions to Prevent Oxidative Damage following Ischemia/Reperfusion

Simón Quetzalcoatl Rodríguez-Lara,[1] Ernesto German Cardona-Muñoz,[1] Ernesto Javier Ramírez-Lizardo,[1] Sylvia Elena Totsuka-Sutto,[1] Araceli Castillo-Romero,[2] Teresa Arcelia García-Cobián,[1] and Leonel García-Benavides[1]

[1] *Instituto de Terapéutica Experimental y Clínica, Departamento de Fisiología, CUCS, Universidad de Guadalajara, Calle Sierra Mojada 950, Colonia Independencia, 44340 Guadalajara, Jal, Mexico*
[2] *Laboratorio de Neurofisiología, Departamento de Fisiología, CUCS, Universidad de Guadalajara, Calle Sierra Mojada 950, Colonia Independencia, 44340 Guadalajara, Jal, Mexico*

Correspondence should be addressed to Leonel García-Benavides; drleonelgb@hotmail.com

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Ischemia/reperfusion (I/R) lesions are a phenomenon that occurs in multiple pathological states and results in a series of events that end in irreparable damage that severely affects the recovery and health of patients. The principal therapeutic approaches include preconditioning, postconditioning, and remote ischemic preconditioning, which when used separately do not have a great impact on patient mortality or prognosis. Oxidative stress is known to contribute to the damage caused by I/R; however, there are no pharmacological approaches to limit or prevent this. Here, we explain the relationship between I/R and the oxidative stress process and describe some pharmacological options that may target oxidative stress-states.

1. Introduction

As early as 1986, Murry et al. [1–3] observed that, after occlusion of the coronary artery and posterior reperfusion, lesions were present in myocardial tissue in the dog, which seemed to accelerate necrotic damage. In addition, histopathological changes in tissues observed at 30–60 min of reperfusion were similar to that observed at 24 h of permanent ischemia. Ischemia/reperfusion (I/R) lesions are present in many diseases that affect multiple health systems [4–8]. The effect of these lesions can range from irreversible damage to death of the injured tissue (e.g., cardiovascular, renal, neuronal, and hepatic) [8].

One of the main events that contribute to this damage is the formation of reactive oxidative species (ROS) and reactive nitrogen species (RNS) and subsequent redox signaling disruption in mitochondria [3, 9–11].

The current therapeutic approaches include pharmacological and mechanical interventions. To date, the mechanical approaches (preconditioning, postconditioning, and remote ischemic preconditioning) have proven to be the most promising; however, these methods are invasive, cannot be used for all cases, and the end results can vary. In contrast, the pharmacological interventions currently available are unable to produce any significant effects on patient prognosis [12–20]. The establishment of animal models of I/R injury has aided in determining the molecular mechanisms involved and possible pharmacological targets.

2. What Is I/R Lesion?

I/R lesions can be defined as a phenomenon that occurs following the block of arterial blood flow to tissue or an organ, which produces a severe imbalance in oxygen and metabolic substrates. This imbalance causes tissue hypoxia and inhibits metabolic processes within cells; paradoxically, the restoration of arterial blood flow and reoxygenation is

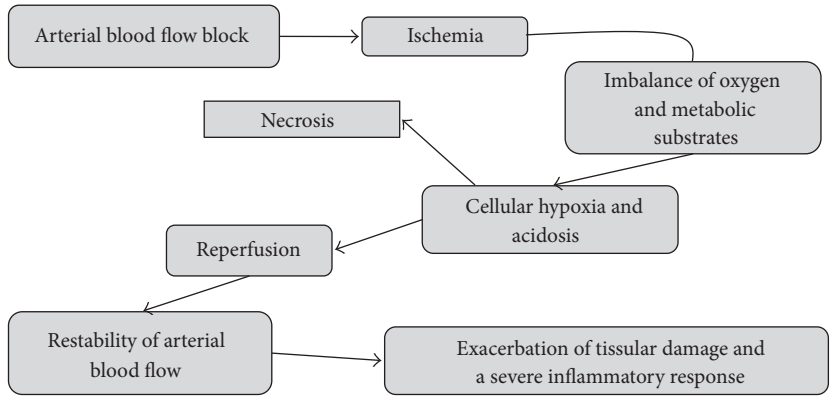


Figure 1: Ischemia reperfusion process. Sequence of stapes and clinical states.

associated with the exacerbation of tissue damage and a severe inflammatory response (Figure 1) [3, 12–22].

3. Mechanism of Lesion

The formation of lesions is caused by multiple events that are triggered by the block of arterial flow and its restoration. The most critical time point in lesion formation is 72 h after reperfusion and the limitation in damage is at 7 days after the initial reperfusion, with recovery taking more than 15 to 90 days [3, 10, 11, 20].

There are eight pathophysiological processes that contribute to lesion formation (Figure 2). These processes can act separately and consecutively, and although their order varies depending on the tissue, they all overlap in one crucial step: permanent mitochondrial lesion and redox signaling disruption [3, 10, 11, 21–25].

4. Imbalance in Metabolic Substrates and Oxygen

Metabolic substrates such as glucose and oxygen are necessary for mitochondrial ATP production. When oxygen concentration is depleted and aerobic glycolysis stopped, cells switch to anaerobic metabolism, causing an increase in lactic acid production that diminishes cytosolic pH. This acid microenvironment within the cytosol helps cells to survive ischemia. Time is an essential component in reaching this imbalanced state and varies depending on the tissue; for example, cardiac cells can tolerate 20 min of ischemia before necrosis and hepatocytes and renal cells more than 30 min, while neuronal cells can tolerate no more than 20 min. Some tissues (e.g., skeletal muscle cells) excel compared with others and can tolerate 2 h of ischemia [2, 3, 13, 15, 53, 54, 64, 65, 91–106].

5. Increase in Cytosolic Cation Levels

During arterial blood flow occlusion (ischemia), cellular metabolism continues. However, the moment an imbalance in cation levels is detected, a series of changes in the cell occurs, which starts with the activation of the Na^+/Ca^{+2} exchanger and L-type Ca^{+2} channels, which cause an increase in the levels of cytoplasmic Ca^{+2}. This increase triggers the activation of Na^+/H^+ exchangers that consequently results in an increase in H^+ and changes the pH of the cell. These events impair the metabolic processes of the cell and affect K^+ channels in mitochondria [21, 23, 33, 64, 100, 101, 107–115].

During the reperfusion process, the cell tries to restore the change in pH by activating the $Na^+/HCO3^-$ exchanger. The efflux of H^+ produces an influx of Na^+ increasing the concentration of Na^+ in the cytosol. This increase activates the Na^+/Ca^{+2} exchanger, which leads to an increase in cytosolic Ca^{+2} [21, 23, 33, 64, 100, 101, 107–115]. The high concentration of Ca^{+2} in the cytosol activates several proteases and other proteins that lead to dysfunction and destruction of organelle membranes and corruption of normal metabolism [21, 23, 33, 64, 100, 101, 107–115].

In myocardial cells, changes in membrane potential traffic activity and water migration secondary to voltage-dependent channel aperture lead to arrhythmia and myocardial stunning. In the kidney, glomerular charge is inverted and overloads filtration solutes, increasing water, protein, and electrolyte loss through urine production. In hepatocytes, membrane potential and pH changes are detected, which suppress and activate many enzymes that optimally operate at a neutral pH, and lead to edema and necrosis [21, 23, 33, 64, 100, 101, 107–115].

6. Mitochondrial Lesions

The metabolic changes in the cytosol following ischemia affect the normal function of mitochondria, which produces an adaptive response brought about by increasing levels of cytosolic Ca^{+2} and decreases in oxygen, NADH, pyruvate, ADP, and Pi. The high concentration of Ca^{+2} activates mitochondrial calcium-sensitive K^+ channels (mtKca) and mitochondrial nitric oxide synthase (mtNOS), which increases the levels of nitric oxide ($NO^\bullet$) (Figure 3) [21, 23, 33, 64, 100, 101, 107–115]. $NO^\bullet$ blocks complex IV in the respiratory chain, inducing an influx of electrons into the mitochondrial matrix and depletion of ATP. $NO^\bullet$ reduces molecules to superoxide anions (O_2^-) and produces high

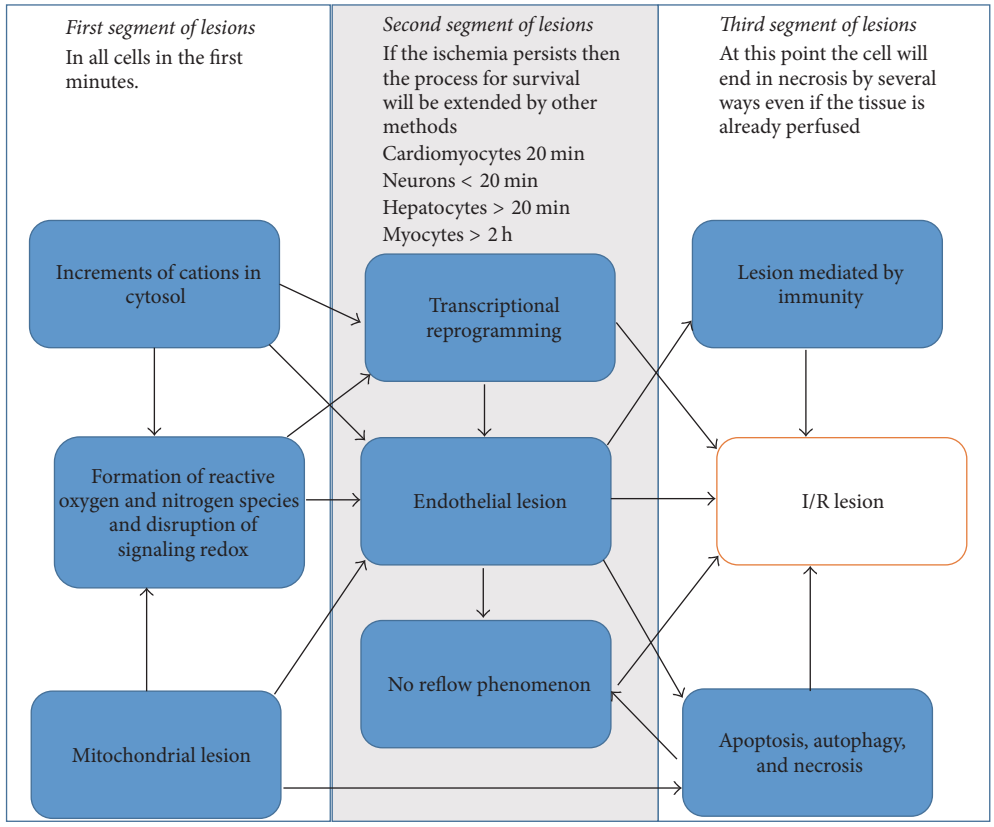


Figure 2: Chronology and correlation of I/R lesion "hot-points." The ischemic process is distinct in each tissue but can be divided into three segments that are shared among all cell types yet show differences in specific details (e.g., timing). The time to damage is prior point.

levels of peroxynitrite ($ONOO^-$). The loss of ATP is caused by the impairment to ATP recycling, depletion of substrates, and inhibition of complex IV in the respiratory chain [21, 23, 33, 64, 100, 101, 107–116]. This influx of electrons into the mitochondrial matrix and the efflux of protons to the cytosol maintains mitochondrial membrane potential; however, it results in an increase in the production of ROS, RNS, and edema and makes the mitochondrial matrix an alkaline environment [21, 23, 33, 64, 100, 101, 107–116].

The ATP depletion induces the activation of mitochondrial ATP sensitive K^+ channels (mtK_{ATP}), resulting in the influx of K^+ to the mitochondrial matrix and efflux of H^+. This change accelerates mitochondrial electron transport in the respiratory chain and produces more influx of electrons to the mitochondrial matrix, which in turn produces more ROS and RNS [21, 23, 33, 64, 100, 101, 107–118].

During reperfusion, the entry of oxygen and metabolic substrates in mitochondria (Figure 3) produces more ROS and RNS. The levels of ROS and RNS and the imminent mitochondrial membrane potential change activate the mitochondrial permeability transition pore (mtPTP), mtK_{ATP}, and mtK_{Ca} dissipating membrane potential and releasing all ROS and RNS [21, 23, 33, 64, 100, 101, 107–118].

This is known as the "point of safe return" (PSR): mitochondria have lost their membrane potential, cell activity is dampened until subsequent death, and the I/R lesion has spread to contiguous cells. These contiguous cells attempt to survive injury but enter the I/R lesion state when ROS, RNS, and other molecules are released from the dying cell. It should be noted that the establishment of any therapeutic prevention must take place before PSR, with these strategies known as preconditioning. Any strategies that take place after PSR are known as postconditioning. The PSR process in mitochondria is present in all cells, but the ability to adapt to injury is tissue dependent. This is because of the high levels of oxidative stress (OS) in different tissues, and their mechanisms for adapting to sudden microenvironmental changes [21, 23, 33, 64, 100, 101, 107–118].

7. What Is Oxidative Stress?

OS refers to an imbalance between the prooxidant and antioxidant levels, in favor of the prooxidants, in cells and tissues. These changes lead to modification or damage to lipids, proteins, and DNA. Prooxidants cause or promote oxidation. Antioxidants are molecules that inhibit the formation of prooxidants and inhibit oxidation [33, 66, 107, 119–126].

8. Formation of Free Radicals

The major source of free radicals in I/R is mitochondria [21, 23, 33, 66, 107, 108, 111, 112, 119–127]. Normally, the electron transport mechanism in the mitochondrial respiratory chain

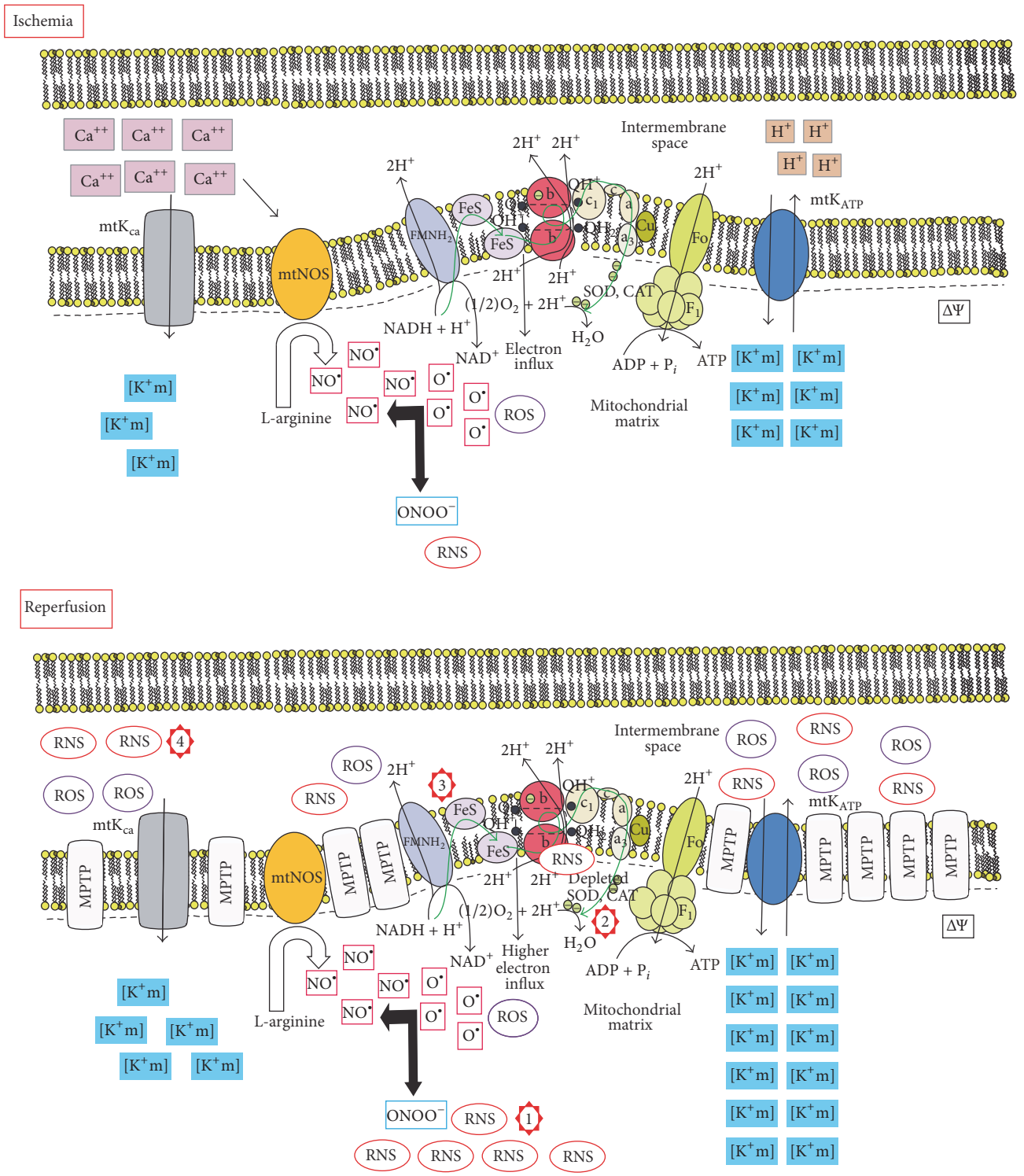


Figure 3: Mitochondrial I/R process. Numbers indicate the four major therapeutic points. (1) Production of ROS and RNS during I/R; (2) depletion of scavenger enzyme systems that reduce free radical; (3) overproduction of superoxide during reperfusion; (4) release and disruption of redox signaling. See the text for a more detailed description on the physiopathological processes.

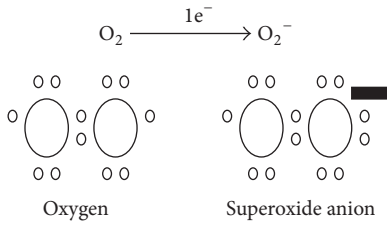


Figure 4: Formation of superoxide anion. The two molecules of oxygen, which are in equilibrated energetic form (6 electrons each one), accept one electron in the last orbital which leads to unstable energy form (7 and 8 electrons), making the molecule of oxygen need to take one electron from the environment to be in its energetic equilibrium form again.

is impaired, this produces ROS from one-electron reduction of oxygen (see (1)) [33, 66, 107, 119–126, 128].

$$O_2 \xrightarrow[2H^+]{1e^-} O_2^- \xrightarrow{1e^-} H_2O_2 \xrightarrow{1e^-} {}^{\bullet}OH \xrightarrow{1e^-} H_2O \qquad (1)$$

is the metabolic process of reduction of free electron. Observe the production of 4 free electrons that must be reduced to water and one diapered electron.

The lifespan of O_2^- (Figure 4) in biological systems is less than a second (50 μs) and has a diffusion distance of ~320 nm. It rapidly reacts with another molecule of superoxide to form hydrogen peroxide (H_2O_2) [33, 66, 107, 119–126, 129, 130].

In mitochondria, one of the important reactions is the diffusion reaction between O_2^- with $NO^{\bullet}$ (termed the radical-radical reaction) (see (2)) to form $ONOO^-$, which can diffuse across biological membranes at a 400 times greater rate than O_2^-. The half-life of $ONOO^-$ is <0.1 s and it has high reactivity with organic molecules, especially lipids [33, 66, 107, 119–126, 129, 130].

$$O_2^- + NO^{\bullet} \longrightarrow ONOO^- \qquad (2)$$

is radical to radical reaction to form peroxynitrite on one of the most instable radicals.

Under physiological states, the production of O_2^- produces H_2O_2 via manganese superoxide dismutase (MnSOD) in the mitochondrial matrix. This enzyme is found in tetramers, with each subunit consisting of 151 amino acids. MnSOD maintains the steady-state concentration of O_2^- at 10-10 M during acute phases of ischemia, but when this phase is over, the activity of MnSOD increases, which results in the production of massive levels of O_2^- that are reduced by $NO^{\bullet}$. Because of membrane potential, this molecule stays in the mitochondrial matrix. During reperfusion, the respiratory chain accelerates and O_2^- is overproduced. MnSOD competes with $NO^{\bullet}$ to reduce the amount of O_2^- and consequently forms more $ONOO^-$, which is released into the cytoplasm via the mtPTP [33, 66, 107, 119–126, 129, 130].

9. How Free Radicals Can Cause Damage to Cells?

The free radicals "take" one electron from the adjacent molecule, which leads to the formation of a new free radical that will "take" one electron from the adjacent molecule. Therefore, a chain reaction occurs that will only end when the free radicals are reduced by antioxidants [25, 123, 125, 126, 128, 130].

10. Damage to Lipids in I/R

The interaction between OS and lipids is one of the most prevalent causes of cellular injury. The degradation products of lipid peroxidation are aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and hydrocarbons such ethane and ethylene. Lipid peroxidation in mitochondria is particularly cytotoxic and has multiple effects on enzyme activity and ATP production, as well as on the initiation of apoptosis [107, 110, 115, 116, 119, 122–125, 130].

11. Damage to Proteins in I/R

Damage to proteins occurs through site specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charges, and increased susceptibility to removal and degradation. The activity of $ONOO^-$ produces nitrotyrosine; meanwhile O_2^- inactivates enzymatic function [107, 110, 115, 116, 119, 122–125, 130].

12. Damage to DNA in I/R

OS can induce numerous lesions in DNA that cause deletions, mutations, and other lethal genetic effects. The sugar and the base fraction are susceptible to oxidation causing base degradation, single-strand breakage, and cross-linking to proteins. One product of DNA damage is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-Oxo-dG) [107, 110, 115, 116, 119, 122–125, 130].

13. Pathways Affected by Redox Signaling in I/R

Redox signaling describes the action of ROS and RNS on altering intrinsic cellular activity. At low concentrations ROS and RNS work as signaling molecules, while at high concentrations they damage multiple structures, especially mitochondria [23–25, 67, 119, 122, 129, 131].

The mechanism by which OS alters protein function and structure involves redox-reactive cysteine residues on proteins. Oxidation of these residues forms reactive sulfenic acid, which in turn forms disulphide bonds with nearby cysteines, and undergoes further oxidation into sulfinic or sulfonic acid, or sulfenamide when nitrogen is present locally. These redox modifications are reversible through reducing systems such as thioredoxin and peroxiredoxin [23–25, 67, 119, 122, 129, 131].

14. The Mitogen-Activated Protein Kinase Cascade in I/R

The mitogen-activated protein kinase (MAPK) cascade consists of a three-rung kinase tier. The canonical cascade occurs

when MAPK kinase kinases (MAPKKK) phosphorylate and activate MAPK kinases (MAPKK), which phosphorylate and activate MAPKs [23, 132–139].

There are two noncanonical pathways: the apoptosis signal-regulated kinase 1 (ASK1) pathway and cGMP-dependent protein kinase (PKG) pathway. In the former MAPK cascade, ASK1 is an upstream MAPKKK that regulates c-Jun N-terminal kinases (JNK) and p38 kinase (p38), leading to apoptosis via phosphorylation of MKK4, MKK3, and MKK6 MAPKKs. ASK1 is activated by OS and mediates p38 signaling, which leads to differentiation and immune signaling. However, activation of ASK1 by high levels of OS (via oxidization of two cysteine residues in the redox center of thioredoxin) induces ASK1 dissociation and allows its complete oligomerization with tumor necrosis factor-α receptor associated factor (TRAF) and ASK2, thereby promoting cell death. Alternatively, the PKG pathway is integrated by PKG1α, protein kinase A (PKA), and protein kinase C (PKC) and, similarly, is also regulated by a redox mechanism [23, 132–139].

15. The Phosphoinositide 3-Kinase Signaling Pathway in I/R

Phosphoinositide 3-kinase (PI3K) consists of one catalytic (p110) and one regulatory (p85) subunit and is firmly coupled with the receptor tyrosine kinase (RTK) family, which are activated by several growth factors. PI3K catalyzes phosphatidylinositol 4,5-diphosphate (PIP2) to synthesize the second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 serves as a membrane-bound signaling molecule that recruits proteins containing pleckstrin homology (PH) domains, such as phosphoinositide-dependent protein kinase (PDK) and protein kinase B (AKT) serine threonine/kinase, which mediate further downstream signaling events. Phosphatase and tensin homolog (PTEN) phosphorylates PIP3, causing inhibition and ensuring that the PI3K pathway is subject to reversible redox regulation by OS. Oxidation of PTEN by OS leads to persistent activation of the PI3K pathway, causing permanent activation of RTKs [14, 19, 134, 140–146].

16. The Redox Factor-1 and NF-E2-Like 2 (Nuclear Factor Erythroid 2) Pathway in I/R

Redox factor-1 (Ref-1) is a multifunctional protein that regulates transcription factor activity and also mediates base excision repair. The transcriptional regulatory function of Ref-1 is exerted by its redox activity on several transcription factors, including activator protein 1 (AP-1), p53, nuclear factor kappa B (NF-κB), and hypoxia inducible factor 1 (HIF-1).

Ref-1 activates the AP-1-Fos-Jun complex via redox regulation of cysteine residues in Fos-Jun DNA binding domains. As it mediates both DNA repair and redox activation of key transcription factors involved in cellular defense (including AP-1 and NF-κB), upregulated Ref-1 activity protects DNA from oxidative damage. Consequently, OS conditions can activate detoxification genes such as glutathione S-transferase (GST), NADPH quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO1), and ferritin H (FH) [23–25, 67, 119, 122, 129, 131].

NF-E2-like 2 (Nrf2) is another transcription factor, which activates antioxidant responsive element- (ARE-) dependent transcription of target genes under OS. These genes serve as antioxidants in processes such as electrophile detoxification, glutathione synthesis, and ROS homeostasis [23–25, 67, 119, 122, 129, 131, 147].

The interaction between these two pathways has a protective, synergistic effect against OS. During I/R, Ref-1 and Nrf2 upregulation increases expression of NF-κB, which increases apoptosis and inflammation [23–25, 67, 119, 122, 129, 131, 147, 148].

17. Transcriptional Reprograming in I/R

The alteration in transcriptional control of gene expression induced by I/R lesions is known as transcriptional reprograming. Oxygen depletion and increased ROS, RNS, and apoptosomes lead to transcriptional alterations within the nucleus. Damage (secondary to redox signaling disruption) causes toll-like receptor (TLR) expression in the membrane of affected cells, indirectly affects NF-κB expression, and increases MAPK and interferon activity. TLR3 is overexpressed in necrotic cells, TLR2 is overexpressed in hypoxic and inflammatory states, while TLR4 is exclusively overexpressed in renal I/R lesions. During the process of adaption, cells affected by I/R lesions show specific expression of microRNAs (miRNAs), which modulate gene expression through transcriptional and posttranscriptional pathways. For example, miRNA-21 blocks PTEN expression in the ischemic state and reduces apoptosis in the first 48 h but shows persistent overexpression during this time and subsequently increases apoptosis as a result of PTEN reduction. Moreover, miRNA-378 blocks caspase-3 expression and reduces apoptosis in the reperfusion state. Expression of multiple genes depends on the amount of OS, time of ischemia, and number of necrotic cells produced during ischemia [10, 11, 19, 22, 23, 93, 98, 132–139, 149–170].

18. Apoptosis, Autophagy, and Necrosis in I/R

The process of cellular destruction starts after mitochondrial lesion, disruption of redox signaling, and transcriptional reprograming. During apoptosis and after PSR is reached, the mtPTP releases cytochrome C from the membrane, activating the caspase cascade and pannexin hemichannels, which release ATP and work as beacons for phagocytic cells. Redox signaling disruption and transcriptional reprograming lead to NF-κB activation, which activates apoptosis and is histologically characterized by nuclear fragmentation, endosome formation, mitochondrial and cellular contraction, and loss of membrane potential. Autophagy is also produced as an adaptive response to sublethal OS, with metabolic change and transcriptional survival reprograming in endosomes, loss of organelles, and formation of coil-shaped vacuoles

observed. Cell damage can progress to necrosis, with or without the presence of edema within cells and organelles, or cause cell membrane disruption and efflux of enzymes to the extracellular space [10, 11, 19, 22, 23, 93, 98, 132–139, 149–176].

19. Immunity-Mediated Lesions in I/R

During the I/R lesion, three pathways are activated: sterile inflammation, adaptive response, and innate autoimmunity. Sterile inflammation is mediated by TLRs, which lead to NF-κB, MAPK, and interferon activation. These receptors also produce chemotaxis of inflammatory and phagocytic cells, which start the inflammatory response. After 24 h of reperfusion, the adaptive response begins, with expansion and recruitment of T cells. At 72 h, the highest level of response and T_{reg} depletion is reached. This affects the innate response, leading to autoimmunity characterized by autoantibody formation by B cells, complement activation, and Bcl3 depletion, which inhibits granulopoiesis production [10, 11, 22, 97, 152, 177–180].

20. Endothelial Lesion

Endothelial lesions that present during the ischemic state are principally affected by decreased oxygen, increased ROS and RNS, and redox signaling disruption, followed by membrane potential loss and increasing membrane permeability, chemotaxis, and imbalanced capillary vasoconstriction/vasodilatation factors. In the reperfusion state, the endothelium suffers lesions caused by the immune response and activation of the coagulation system, increasing leucocitary adherence, and platelet-leucocitary interaction. This process is produced by mechanical brushing that occurs in the damaged endothelium and causes e-selectin adherence protein expression in the membrane, which interacts with the ligand, selectin-1, and in neutrophils coactivates integrin-$\alpha\mu\beta 2$. Following interaction and formation of this complex, neutrophils are able to bind to erythrocytes and platelets through their membrane, which directs them to the damaged tissue and increases the inflammatory response in affected tissue [10, 11, 22, 117, 127, 180, 181].

21. No Reflow Phenomenon

The no reflow phenomenon is present in the reperfusion state because of endothelial cell injury, activation of the coagulation process, and increased leucocitary adherence. This phenomenon increases impedance of microvascular flow and capillary occlusion by leucocytes and is present in 60%–68% of all I/R cases [9–11, 22, 91, 182].

22. Analysis of the Pharmacological Approach in I/R

The actual pharmacological approach to prevent or mitigate I/R lesions has so far been unsuccessful. This is likely because one drug cannot cure all disturbances, and, indeed, the phenomenon is a result of several events that follow a specific sequence. Consequently, attempting to cover the physiopathological process with just one medication is likely not enough. Most studies have attempted to show that one drug will make a difference, and regarding I/R lesion in vitro experiments and preclinical studies have shown good results. Nonetheless, the lesion is manifested systemically and affects several processes; therefore, the correct approach needs several drugs targeting the physiopathological process (Figure 3) [1–3, 55, 64, 65, 75, 92, 94, 95, 100, 104, 183–185].

23. The Gender Aspect of I/R

Recent studies dealing with survival in specific pathological conditions related to I/R have shown significantly better patient survival in females than males. However, other studies have shown a much poorer outcome associated with female patients. The reason for these controversial results is not clear, but a variety of different factors may influence interpretation of these studies. For example, age (postmenopausal), race, underlying disease, and/or medications may impact the outcome. Failure to account for and control these different variables makes it difficult to accurately assess the role of gender in reducing or increasing survival expectancy following I/R lesions [186–191].

24. The Physiopathological Approach

The critical points in OS in I/R are the production of prooxidants, the depletion of SOD, the accumulation of free radicals, loss of mitochondrial membrane potential with subsequent release from mitochondria, and redox signaling disruption. There are several drugs that may have therapeutic potential (Table 1).

25. Electron Acceptor

The process of reducing free radicals is made possible based on the structure of the molecules. These drugs are equipped by one or more aromatic ring with hydroxyl groups in their structures. Therefore, they can exchange the free radical with the hydroxyl group and end the chain reaction of free radical accumulation. The structure of curcumin and cannabidiol (Figure 5) has a typical antioxidant architecture that confers no enzymatic scavenging ability [24, 26–29, 33–35]. Besides, the cannabidiol in the liver and cardiac I/R lesion has shown that it could interact directly with the cannabinoid CB_2 receptor. In the heart the agonism of the CB_2 receptor was shown to modulate the myocardial inflammation and attenuates the infarct size (142) and decreases the myocardial ROS and RNS generation, restores the glutathione content and SOD activity, and modulates the signaling redox and the NF-κB activation (143). In the liver the cannabidiol attenuates tissue oxidative and nitrative stress, acute and chronic hepatic inflammatory response, signaling redox, and cell death by its strong antioxidant ability and the interaction with the CB_2 receptor (144).

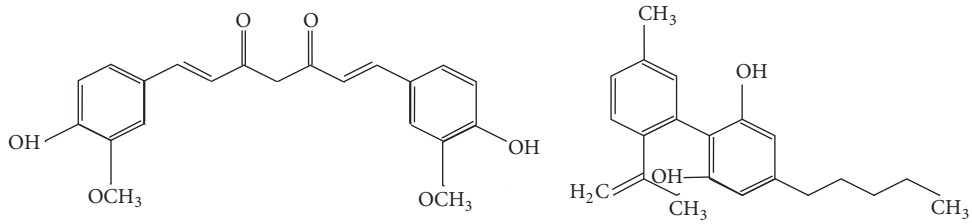
26. Block of Mitochondrial Respiratory Chain Complex I

Complex I in the mitochondrial respiratory chain is the most important for the production of O_2^-, which is produced by

Table 1: Promising drugs in the I/R lesion.

Drug	Molecular mechanism	Beneficial effect in IR	Adverse effects	Organ cell major effect	Literature support study
Curcumin	Electron acceptor	Reduction of free radicals	Dermatitis Bitter taste	Neurons	[26–32]
Cannabidiol	Electron acceptor	Reduction of free radicals, modulation of inflammation and signaling redox	Neuronal disorders	Neurons Hepatocyte Cardiomyocyte	[24, 33–43]
Metformin	Block of complex I in respiratory chain	Modulated production of free radicals	Lactic acidosis Blood coagulation disorder Liver function test abnormal Encephalopathy	Hepatocyte Cardiomyocyte Neurons	[44–52]
Pioglitazone	*PPAR-γ agonist	Increments on the expression of **MnSOD and some other survival gens	Congestive heart failure Edema Liver function test abnormal Osteopenia	Endothelial cells Cardiomyocyte Hepatocyte	[50–63]
Atorvastatin	Pleiotropic effects	Reduction of free radicals, increments on expression of MnSOD, modulation of survival gens	Diabetes mellitus Reduction of ubiquinone level Liver function test abnormal Autoimmune disease Rhabdomyolysis Acute kidney injury	Endothelial cells Cardiomyocyte Hepatocyte Musculoskeletal	[30–32, 64–74]
Telmisartan	Pleiotropic effects	Reduction of free radicals, increments on expression of MnSOD, modulation of survival gens	Angina pectoris Edema Carcinogen effect Liver function test abnormal	Endothelial cells Cardiomyocyte Hepatocyte	[58, 75–90]

*PPAR-γ: peroxisome proliferator activated receptor-γ. **MnSOD: manganese superoxide dismutase.



Curcumin Cannabidiol

Figure 5: Structure of curcumin and cannabidiol, with properties to interact with free radicals in their proximity.

the reverse transport of electrons from complex II. During the ischemic phase, the partial disablement of this primary component results in free radical formation because of the strain it has on the electron transport to complex III, and, during the reperfusion phase, the production of O_2^- is attenuated [36–38, 44]. One of the actions of complex I inhibitor metformin is to induce mild and specific inhibition of mitochondrial respiratory chain complex I, which reduces free radical formation [44].

27. Peroxisome Proliferator Activated Receptor-γ (PPAR-γ) Agonist

This class II nuclear receptor interacts with multiple survivor genes and can down- or upregulate proteins involved in the tolerance to I/R injury. Normally, PPAR-γ works as a heterodimer with retinal receptor but interacts with multiple response systems in the DNA; activation of this receptor can increase the expression of MnSOD and other enzymatic

scavengers and blocks the induction of apoptosis. The PPAR-γ agonists pioglitazone and telmisartan increase the bioavailability and action of PPAR-γ to improve cell survival [45–47, 53–55, 76–78, 192–194].

28. Pleiotropic Effects

There are multiple pathways involved in the production of OS that can be modulated, and several in vitro studies and animal models have shown promising results. In vitro experiments using atorvastatin have shown a reduction in ROS and RNS levels in I/R and various injury models including degenerative pathologies and chronic diseases. In addition, atorvastatin was shown to activate nuclear receptors such PPAR-γ. Telmisartan has a similar effect and reduces OS by activating PPAR-γ, blocking the angiotensin II receptor, type 1 (AT1 receptor), increasing levels of enzymatic scavengers, and activating cell survival pathways [45–47, 53–58, 65–68, 76–78, 192–194].

29. Prejudice in the Physiopathological Approach

The adverse effect of drugs may influence the therapeutic effect, with possibly the most questionable drugs being atorvastatin (statins) and pioglitazone (PPAR-γ agonist). The atorvastatin controversy owes to the associated increased risk of diabetes mellitus, with a meta-analysis showing that, after at least 4 years of treatment, patients have an incidence of 5%-6% for diabetes mellitus onset and decrease in serum ubiquinone levels of 32%–54% during statin use for at least three months. However, treatment for I/R lesions last for only a short period of time and should be administered during the first hours of diagnosis and for not longer than two weeks, to reduce the chance of suffering major adverse effects. It is well known that pioglitazone should not be used in class II or III New York Heart Association heart failure scale patients, or those with a depressed ejection fraction of less than 40%, due to exacerbation of congestive heart failure (approximately 9%) in studies when the treatment time lasted from 3 weeks to 3 months using the maximal recommended dose. Treatment with pioglitazone for I/R lesions should not surpass the recommended dose or last longer than two weeks, as mentioned above.

30. Future Prospects

Several attempts have been made to inhibit I/R lesions, but the real challenge lies in attenuating the processes that lead to the formation of lesions, which include the mitochondrial production of ROS and RNS and disruption of signaling redox. To date, most research has focused on the inflammatory response [96, 97], and there is limited knowledge on the effect of preconditioning, postconditioning, and remote ischemic preconditioning. The most studied therapeutic approaches with respect to I/R lesions are the mechanical process of preconditioning, postconditioning, and remote preconditioning [1, 2, 10, 12, 13, 15–18, 20, 21, 64, 92, 93, 108, 109, 114, 118, 142, 195–204]; however, the results are controversial, and the greatest benefits have only been observed in animal models [2, 10, 64, 197]. In addition, meta-analysis and randomized trials have shown that this procedure has no beneficial effect on mortality but can improve periprocedural myocardial infarction and afford some neuroprotection [196, 203, 204]. However, it should be noted that this procedure needs to be performed within a strict timeframe and is dependent on the condition of the patient. Therefore, not all patients are suitable candidates. To complicate matters, the heterogeneity of injury also limits the effectiveness of this method. These variables have biased the statistical evaluation of this approach. Nevertheless, no one has implemented a pharmacological regimen for the management of OS, before, during, or after the procedure despite all the evidence for the involvement of OS.

31. Conclusion

OS in I/R lesions has a big impact on the activation of multiple secondary mechanisms of damage. Therefore, the search for a therapeutic pharmacological regimen that can inhibit the production of ROS and RNS and can modulate the signaling redox should take priority in the treatment of I/R lesions. There are currently no studies on pharmacological regimens at any institution or clinical trials for patients who suffer the I/R phenomenon in combination with other strategies of mechanical procedure. The establishment of new animal models and studies focusing on the mechanism of action of known drugs that have been used for other pathological states has revealed that reducing OS can provide beneficial outcomes. Perhaps combination therapy could attenuate OS further and provide a better prognosis for patients who suffer from this phenomenon following mechanical procedures. There is no sufficient data to confirm or reject this hypothesis. Therefore, more studies should be performed to determine a standardized therapeutic regimen to control or prevent the OS damage in I/R before, during, and after mechanical procedures.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Chlorinated Phospholipids and Fatty Acids: (Patho)physiological Relevance, Potential Toxicity, and Analysis of Lipid Chlorohydrins

Jenny Schröter and Jürgen Schiller

Institute for Medical Physics and Biophysics, Faculty of Medicine, Leipzig University, Leipzig, Germany

Correspondence should be addressed to Jenny Schröter; jenny.schroeter@medizin.uni-leipzig.de

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Chlorinated phospholipids are formed by the reaction of hypochlorous acid (HOCl), generated by the enzyme myeloperoxidase under inflammatory conditions, and the unsaturated fatty acyl residues or the head group. In the first case the generated chlorohydrins are both proinflammatory and cytotoxic, thus having a significant impact on the structures of biomembranes. The latter case leads to chloramines, the properties of which are by far less well understood. Since HOCl is also widely used as a disinfecting and antibacterial agent in medicinal, industrial, and domestic applications, it may represent an additional source of danger in the case of abuse or mishandling. This review discusses the reaction behavior of in vivo generated HOCl and biomolecules like DNA, proteins, and carbohydrates but will focus on phospholipids. Not only the beneficial and pathological (toxic) effects of chlorinated lipids but also the importance of these chlorinated species is discussed. Some selected cleavage products of (chlorinated) phospholipids and plasmalogens such as lysophospholipids, (chlorinated) free fatty acids and α-chloro fatty aldehydes, which are all well known to massively contribute to inflammatory diseases associated with oxidative stress, will be also discussed. Finally, common analytical methods to study these compounds will be reviewed with focus on mass spectrometric techniques.

1. Introduction

Inflammation, as an adaptive response to noxious stimuli such as infection or tissue injury, is an important and complex physiological process mediated by the immune system [1]. In general, a homeostatically controlled inflammatory response is a beneficial "weapon" of the organism to "fight" against infections and infiltrating microorganisms such as bacteria, but a tissue malfunction or homeostatic imbalance in favor of proinflammatory mediators can become very harmful to mammals due to the permanent stimulation of immune cells such as granulocytes or macrophages. A dysregulated immune response may lead, for instance, to tissue degeneration as observed in the loss of cartilage during rheumatoid arthritis [2]. Neutrophilic granulocytes (a crucial cellular part of the immune system) activated during chronic inflammation generate not only proteolytic enzymes and antibacterial proteins but also considerable amounts of reactive oxygen and nitrogen species (ROS, RNS) including both nonradical species (hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$)) and different free radicals, which are, transient compounds with at least one unpaired electron (superoxide anion ($O_2^{\bullet -}$), nitric oxide ($NO^\bullet$)) [3]. It is important to note that common atmospheric oxygen ("triplet oxygen", O_2) already represents a diradical ($^\bullet O\text{-}O^\bullet$) where the spins of the valence electrons are in parallel orientation. This $^3\Sigma g$ state is rather stable and explains why oxygen undergoes reactions only at elevated temperatures or under enzyme catalysis. This behavior changes significantly if singlet oxygen is considered: in this state ($^1\Sigma g$) oxygen is much more reactive due to the antiparallel orientation of the valence electrons. A more detailed discussion of these aspects is, however, beyond the scope of this paper. Although oxygen is needed by all aerobic organisms, its concentration has to be tightly regulated: the replacement of atmospheric air by pure (100%) O_2 would be strongly toxic for living organisms. Animals used for

experimental purposes die after a few days in a pure oxygen atmosphere as a result of excessive oxidation reactions, which cannot be compensated by the organism. It has also been shown that the tumor incidence increases if the oxygen partial pressure in the air exceeds only slightly the standard value of about 21% [4].

1.1. Generation of HOCl and Other Reactive Oxygen Species. O_2 is converted by a number of different enzyme-triggered reactions into even more reactive compounds. In the first step, O_2 is converted into superoxide ($HOO^{\bullet}$), which exists at physiological pH primarily in the form of the deprotonated superoxide anion radical ($O_2^{\bullet-}$). These radicals dismutate either spontaneously or particularly in the presence of the enzyme superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2). The enzyme myeloperoxidase (MPO) converts H_2O_2 and chloride ions into HOCl, a highly reactive chlorine species (RCS) reacting with the majority of biologically relevant compounds.

HOCl (including its salt sodium hypochlorite (NaOCl)) as a molecular (2-electron) agent has a strong oxidizing and chlorinating ability to ensure high bactericidal and cytotoxic properties. This makes HOCl one of the most powerful in vivo oxidants. Accordingly, HOCl is also used as bleaching, disinfecting, and antiseptic agent for medicinal, industrial, and domestic applications [5]. Normally, the commercially available hypochlorite solution is more stable and much less reactive than the free acid (HOCl). However, both species are (due to the pK value of about 7.53) nearly equally concentrated at physiological pH (7.4). This implies that typical household cleaners (such as "Chlorix") can be as harmful as the in vivo generated HOCl in the case of abuse or mishandling [6]: damages of virtually all relevant biomolecules as well as many tissues may be induced [7]. Some of these generated so-called disinfection by-products show evidence for their cytotoxic, genotoxic, teratogenic, and carcinogenic potential and this important aspect was recently reviewed [8–10]. Some typical pathological and toxic adverse effects in the case of abuse or mishandling of industrial or medicinal used HOCl are summarized in Table 1 and clearly indicate the sensitivity of health tissues to oxidative stress.

1.2. Reactivity of HOCl with Lipids. In contrast to free radicals such as hydroxyl radicals ($HO^{\bullet}$) and nitric oxide ($NO^{\bullet}$), HOCl is characterized by a more selective reaction behavior. We will discuss the HOCl-induced changes of lipids [11] focusing on the phospholipids (PLs) shown in Figure 1.

The majority of PLs consist of a glycerol backbone, esterified with two varying organic fatty acids and one molecule of phosphoric acid. The resulting phosphatidic acid (PA) is again able, via ester condensation with different alcohols, to form a large variety of PL species like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as neutral but zwitterionic representatives or phosphatidylserine (PS) as a typical example of a negatively charged PL. There are also ether PLs: the most important ones are the "plasmalogens" (often from PC and PE species) which possess a vinyl ether in the *sn*-1 position and can be formally considered as the reaction products of a fatty aldehyde with a hydroxyl group of the glycerol.

According to the high amount of PLs in biological membranes, they are important targets for peroxidation by free radicals or oxidation/chlorination by enzymatically generated agents. Those PLs, especially, containing highly unsaturated fatty acyl residues (particularly arachidonic acid), vinyl ether linkages, and/or reactive polar head groups (as in PE or PS) are most vulnerable to oxidation processes [12]. It is generally accepted that oxidized/chlorinated PLs exhibit significant effects in acute and chronic inflammation [13] and are thus associated with inflammatory diseases such as rheumatoid arthritis, neurodegenerative diseases, atherosclerosis, diabetes, systemic lupus erythematosus, and lung infections (Figure 2) [7, 14].

Since oxidized PLs exist in a tremendous diversity in biological samples, their detection in crude mixtures is still challenging. To overcome this problem, many different analytical approaches, in particular mass spectrometry (MS) based methods, were developed in the last decades and can now be considered as the most powerful tools in the field of "lipidomics." "Lipidomics" is a relatively new "omics" approach which focuses on the identification of key species in the lipid metabolism, lipid biomarkers, and lipid signaling alterations in both health and disease [15]. This aspect will be discussed later in this review. However, it has to be emphasized already here that some lipid oxidation products represent transient compounds which either decay into other (more stable) products or react with other biomolecules, which makes the analysis of (at least) some lipid oxidation products a challenging task. Furthermore, the oxidation/chlorination of free fatty acids (FFAs) and especially polyunsaturated fatty acids (PUFAs) by ROS leads also to an enormous pool of bioactive lipid mediators. The most important and intensively investigated classes are the eicosanoid mediators, which are involved in different biological processes such as bone metabolism, nerve development, wound healing, immune responses, and inflammation [16]. However, FFAs represent detergents and are known to destabilize membranes if they are present in elevated amounts [17]. Since they are known as low abundant transient products with a limited stability, the analysis of oxidized/chlorinated FFAs is even more difficult than the investigation of oxidatively modified PLs [16]. Briefly, the search for new lipid and FFA biomarkers containing oxidized moieties in order to diagnose inflammatory processes at an early stage of disease is only realizable with highly specific and particularly sensitive analytical methods which need to be continuously improved.

In this review, we will focus on the PL and FFA oxidation/chlorination by selected agents with the emphasis on HOCl. Some selected products of PC and PE are illustrated in Figure 3.

We will summarize (i) the potential beneficial properties of HOCl and its chlorinated products (particularly chlorohydrins), (ii) the performed in vitro studies which were particularly aimed to find new biomarkers, and (iii) the predominantly reported negative properties of chlorohydrins and other chlorinated species leading to inflammatory diseases. In the last chapter we will provide an overview of the currently used analytical methods to characterize the

Table 1: Potential adverse effects in case of abuse or mishandling of industrial/medicinal used HOCl solution.

Field of application	Abuse/mishandling/adverse effects	Ref.
Household products		
Weakly concentrated solutions	Vomiting, pain, and dyspnea	[117]
Highly concentrated solutions	Nausea, diarrhea, hypotension, coma, hypernatraemic hyperchloraemic acidosis, convulsion, and cardiorespiratory arrest	[117]
Water disinfection	Oxidation of organic matter, anthropogenic contaminants, and iodide and bromide conversion in rivers, lakes, and groundwater	[118]
Endodontics	Chemical burns, tissue necrosis, neurological complications, and upper airway obstruction	[119]

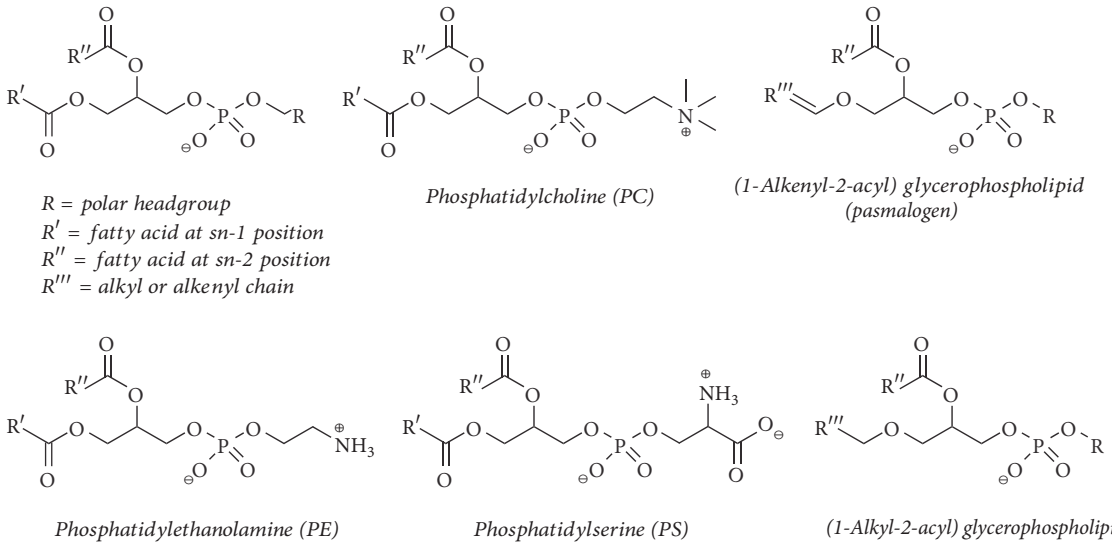


Figure 1: Selected glycerophospholipids (PLs) which will be relevant for this review. Note that phosphatidylcholine (PC) and ethanolamine (PE) are zwitterionic lipids, while PS is an example of a negatively charged PL. PLs in biological samples often contain a saturated fatty acyl residue in *sn*-1 position, while that in *sn*-2 position is normally unsaturated. Ether PL are characterized either by an alkenyl or alkyl linkage in *sn*-1 position at the glycerol backbone. Alkenyl ether lipids are often called "plasmalogens."

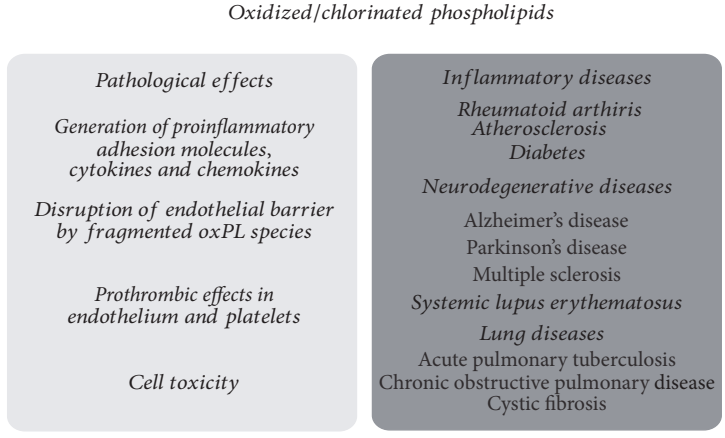


Figure 2: Summary of the known pathological effects mediated by oxidized or halogenated PLs (left) and of the inflammatory diseases in which these lipids are involved (right).

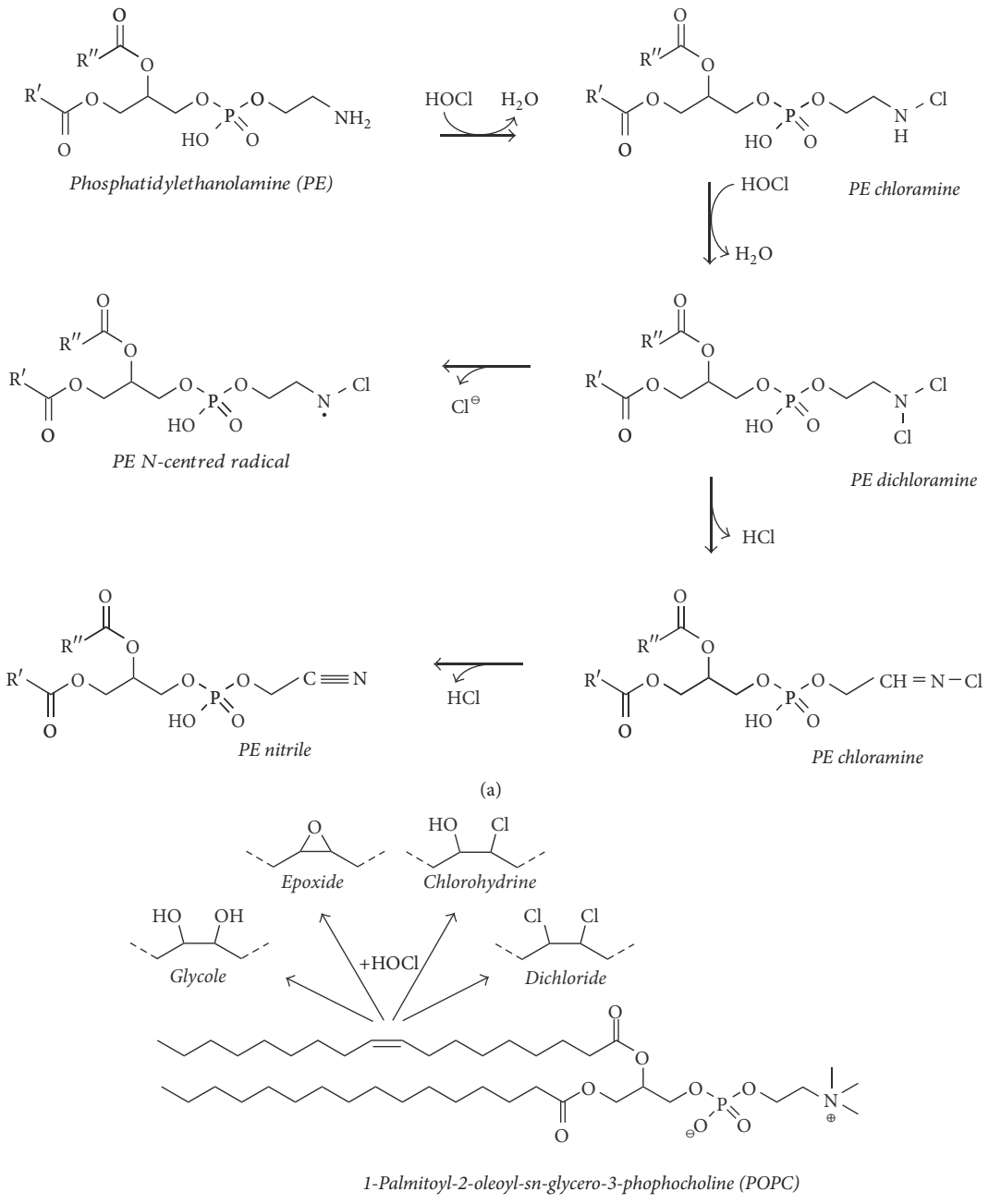


Figure 3: Survey of selected products if an unsaturated PL either with a reactive headgroup (PE, (a)) or with an inert headgroup (PC, (b)) reacts with hypochlorous acid (HOCl). Note the complexity of the reactions and product patterns even if only a single PL is oxidized by HOCl.

oxidized/chlorinated products after PL and FFA treatment with HOCl, focusing on MS methods.

2. In Vivo Generation of HOCl by the MPO-H_2O_2 System

2.1. Release of the MPO-H_2O_2 System by Neutrophils. Neutrophilic granulocytes, which are one subtype of the polymorphonuclear leukocytes (PMNs), are known to play a major role during inflammatory processes. Three types of neutrophilic granules are consecutively generated during the maturation process of the cells: primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules that contain the proinflammatory proteins MPO, lactoferrin, and metalloproteinase 9 (which comprises actually different proteins), respectively [18]. In healthy individuals the release of neutrophils from bone marrow is tightly regulated and known to eliminate pathogens either intra- or extracellularly

via phagocytosis or the release of neutrophil extracellular traps (NETs) [19].

Since the total MPO concentration is about 5% of the whole neutrophil proteins [20], it is one of the major physiologically occurring components in these phagocytes and even increases massively during inflammatory processes. Thus, it is a widely studied enzyme in the fields of inflammation as well as oxidative stress and is often used as a biomarker for many diseases including atherosclerosis, rheumatoid arthritis, and neurodegenerative diseases [21]. The most common diseases linked with the MPO system are summarized in Table 2 and include the changes of the MPO concentration or activity, the investigated matrix, and finally the method by which the MPO amount and/or its activity was determined. For further details see the references provided in Table 2.

During chronic infectious and inflammatory diseases the increased numbers of neutrophils permanently release not only MPO but also ROS during the "respiratory burst" that is triggered by the enzyme NADPH oxidase [22]. This may result, particularly in chronic inflammation, in both immunopathological processes and secondary damages in living organisms [23].

Since rheumatoid arthritis is a widespread disease with an annual incidence of 25–50/100 000 and a prevalence of 0.5–1.0% in North America and Europe [24] and has, even more, a high socioeconomic weight, it is mandatory to find early stage biomarkers to enable early treatment of the disease. The evaluation of the processes of cartilage degradation during inflammatory joint diseases is also a major research topic in our laboratory. Figure 4 shows, exemplarily for all inflammatory processes, the release of ROS (HOCl, H_2O_2, $O_2^{\bullet-}$) as well as antimicrobial enzymes (e.g., MPO, elastase, lysozyme) by an activated neutrophil that infiltrated into the joint space during acute joint inflammation [25]. Symptoms of rheumatoid arthritis may have periods of remission in which the symptoms disappear, alternating with periods of flare-ups, which is correlated with the extent of inflammation.

These cartilage damaging agents do not discriminate between microbial and host target molecules and will thus oxidize a large number of biologically healthy targets such as cells, proteins, or polysaccharides leading to a persistent inflammation. For a detailed description of neutrophilic activity in immune response and inflammation see also [19, 26, 27]. Furthermore, a detailed review about the physiological properties of ROS and their regulatory enzymes has recently been published by Zuo and coworkers [28].

2.2. HOCl Generation during the "Chlorination" Cycle. As a member of the heme peroxidase family in myeloid cells, MPO becomes oxidized at the Fe^{III}-ion position by H_2O_2 under the formation of a short-lived intermediate, *compound I*, with a Fe^{IV} radical cation center [29, 30]. Afterwards, *compound I* is regenerated to the ferric state (three-valent) of MPO, either in two consecutive 1-electron steps (via *compound II*) or by oxidizing Cl^- to HOCl (second order rate constant: $(2.5 \pm 0.3) \times 10^4\ M^{-1}s^{-1}$ [31]) during the chlorination cycle (Figure 5). Further details are available in a detailed review by Arnhold and Flemmig [32].

It is nearly impossible to determine the HOCl concentration under in vivo conditions, as HOCl is continuously generated by MPO (at least if H_2O_2 and Cl^- are present in sufficient amounts) but instantaneously consumed by its reaction with potential target molecules. Therefore, the stationary HOCl concentration is close to zero. However, there are also some papers available which provide dedicated HOCl amounts. Since HOCl is known from these reports to be highly abundant under inflammatory conditions (50–100 mM) [34] and even under physiological conditions (0.34 mM) in the extracellular space [35], it was assumed for many years that it is the only physiologically relevant halogenated product of MPO [36]. Nowadays, it is well accepted that MPO generates, even under physiological conditions, also hypobromous acid (HOBr) and hypothiocyanite (HOSCN), even if the corresponding bromide (20–100 μM) and thiocyanate (20–120 μM) concentrations are much lower than the chloride (100–140 μM) plasma concentrations [31]. Moreover, even cyanate/isocyanate, which are particularly important in human saliva [37], can be generated by MPO, however, to a minor extent [6, 38].

For example, Senthilmohan and Kettle [6] suggested HOBr could be an in vivo relevant major product of MPO at physiological conditions (second order rate constant: $(1.1 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$ [31]) and should be considered if investigations of inflammatory processes are performed. This is surprising, because Br^- is about three orders of magnitude less abundant in comparison to Cl^- (about 10^{-1} versus 10^{-4} M) and implies that bromide is preferentially metabolized by MPO. Furthermore, HOBr can be generated indirectly by the reaction between HOCl and bromide [39] which is also used to synthesize HOBr in the laboratory:

$$Br^- + HOCl \longrightarrow HOBr + Cl^- \quad (1)$$

Both HOCl and HOBr are strongly oxidizing agents with the ability to modify amino moieties in proteins, DNA or PLs (such as PE and PS) resulting in the generation of chloramines and bromamines as primary products [21].

Although a detailed discussion of these aspects is beyond the scope of our review, there are nowadays increasing indications that HOSCN (in particular compared to HOCl) may have protective effects in the organism: HOSCN shows a marked preference for thiol residues, forming products (particularly long-lived sulfenyl thiocyanate (RS-SCN)) that can be "repaired" by the organism [40], resulting in reversible cellular damage. Since HOCl (or HOBr) may react with SCN^- to generate HOSCN (second order rate constant: $(9.6 \pm 0.5) \times 10^4\ M^{-1}\ s^{-1}$) [41] this reaction (2) may represent an important way of how the organism gets rid of excessive HOCl (HOBr)

$$SCN^- + HOCl \longrightarrow HOSCN + Cl^- \quad (2)$$

There is still significant interest in HOCl, since it is a specific product of MPO, whereas HOBr is additionally (or even preferentially) produced by the enzyme eosinophilic peroxidase (EPO) [11]. Due to its high toxicity, HOCl released by neutrophils into the phagosome seems to have a high impact in fighting against microbial pathogens and controlling the

Table 2: Survey of selected diseases where myeloperoxidase (MPO) presumably represents a suitable "biomarker." The alterations of MPO concentration and/or activity are emphasized.

Disease	Changes of MPO	Matrix	Method	Ref.
Arthritis				
Rheumatoid arthritis	Increased activity/concentration	SF	ELISA	[120]
	Increased concentration	Serum	ELISA	[121]
		Plasma, SF	ELISA	[122]
	Increased activity	SF	NMR	[123]
Juvenile idiopathic arthritis	Increased concentration	Serum	Serum	[124]
		Plasma	ELISA	[125]
Atherosclerosis				
	Increased concentration	Plasma	ELISA	[126]
		Mixed with lipoproteins	ELISA	[127]
		AP	IHC; western blot	[128]
			HPLC, western blot	[129]
	Increased release by culprit plaque samples	CP	ELISA	[130]
	Increased activity	AP	Spectrophotometry	[131]
Diabetes				
	Increased level	Serum	Spectrophotometry	[132]
	Increased activity at higher G allele, anti-inflammatory effect of mutant A allele	MPO -463 G/A SNP	PCR	[133]
	Increased concentration	Plasma	ELISA	[134]
	Decreased activity	Neutrophilic cell lysate	Spectrophotometry	[135]
Neurodegenerative diseases				
Alzheimer's disease	Increased concentration	Plasma	ELISA	[136]
	Expression	Astrocytes	Confocal microscopy, IHC	[137]
	Increased Expression	Brain tissue	Immunoblot, IHC, PCR	[138]
Parkinson's disease	Expression	Brain tissue	IHC	[139]
Multiple sclerosis	Increased activity/concentration	Cerebral Cortex	Colorimetric analysis	[140]
	Expression	Microglia	IHC	[141]
Systemic lupus erythematosus				
	Increased concentration	Serum	ELISA	[121]
		Plasma	ELISA	[142]
Lung diseases				
Acute pulmonary tuberculosis	Increased concentration	Serum	EIA	[143]
COPD	Increased levels	Sputum	Meta-analysis	[144]
			ELISA	[145, 146]
Cystic fibrosis	Increased concentration	Sputum	ELISA	[147]

SF, synovial fluid; AP, atherosclerotic plaque; COPD, chronic obstructive pulmonary disease; CP, culprit plaque; EIA, Enzyme Immunoassay; ELISA, Enzyme-Linked Immunosorbent Assay; HPLC, High Performance Liquid Chromatography; IHC, immunohistochemistry; NMR, Nuclear Magnetic Resonance; SNP, Single Nucleotide Polymorphism.

bacterial population in the host [42]. However, the impaired homeostasis between oxidants and antioxidants, in favor of ROS production, leads to an uncontrolled generation of HOCl, which is associated with chronic inflammatory diseases (Figure 2), may have carcinogenic and mutagenic potential [43], and is even known as a stable but highly reactive neurotoxic oxidant in the brain, where HOCl reacts rapidly with H_2O_2, $O_2^{\bullet-}$ or nitrite generating $^1O_2$, $HO^{\bullet}$, or nitryl chloride (NO_2Cl) that may contribute to extensive tissue damage [44]. Due to its fast homeostatic consumption, in vivo generated HOCl cannot be measured directly, but either as stable scavenger products or via the determination of the MPO activity in the corresponding body fluid, for instance, by using the well-known conversion of guaiacol into

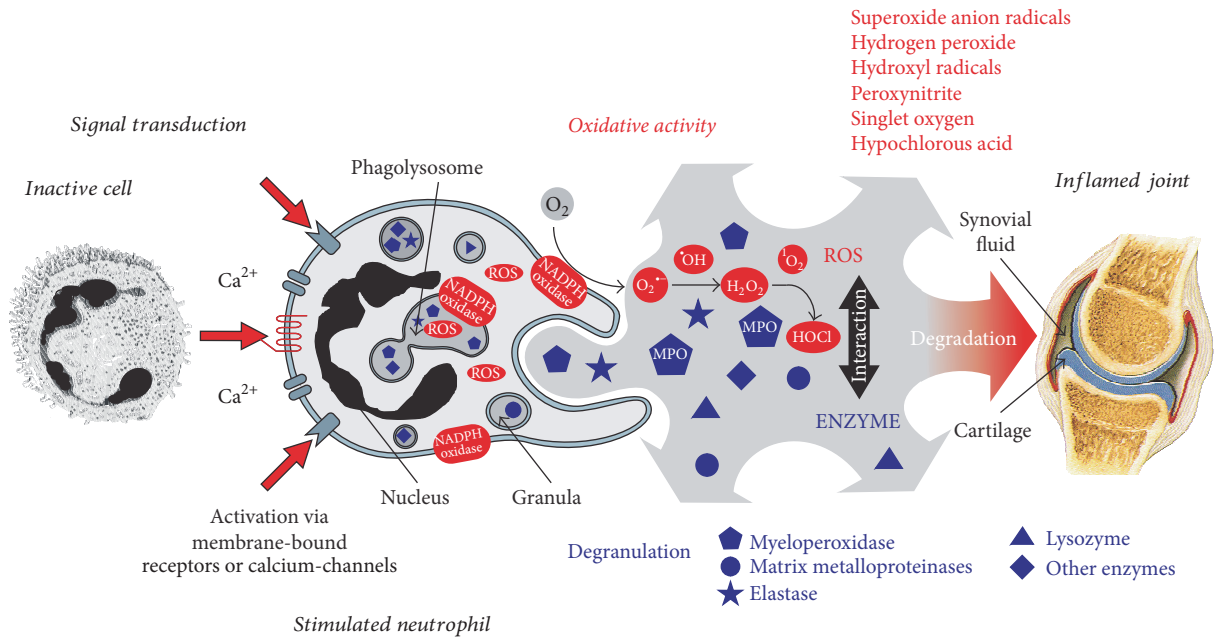
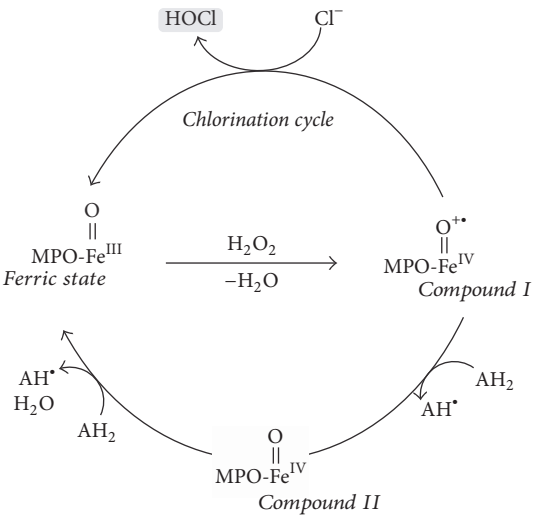


FIGURE 4: Overview of the released reactive oxygen species (ROS) and antimicrobial enzymes by an activated neutrophil during the inflammatory process in an inflamed joint. Due to the release of highly toxic ROS, most of them (including HOCl) do not discriminate between microbial and "healthy" targets, leading to an uncontrolled oxidation of all biomolecules and a persistent inflammation (reprinted from Schiller et al. [25], with permission from Elsevier).



AH_2 = peroxide electron donor

FIGURE 5: Generation of HOCl by MPO via a chlorination cycle. In the first step, MPO is oxidized by hydrogen peroxide (H_2O_2) into the short-lived compound I that contains a Fe^{IV} radical cation center. Compound I is further regenerated to the educt, the ferric state MPO, either by two consecutive 1-electron steps via compound II or by oxidizing Cl^- to HOCl (modified in accordance with [33]).

tetrahydroguaiacol [45]. Therefore, different methods were established to measure stable HOCl-induced products, often by absorbance or luminescence methods, but increasingly also by MS [46]. The optimum biomarker for HOCl should be specific, stable, and sensitive to determine [47]. However, due to the fast turnover of HOCl, it is still a challenge to find a biomarker that combines all these properties.

2.3. Survey of MPO Activity by HOCl Determination. Although HOCl is known to chlorinate a variety of different biomolecules, amino acids are the most susceptible targets for chlorination by HOCl. The concentration of 3-chlorotyrosine (and to a minor extent 3,5-dichlorotyrosine) is often considered as the biomarker of choice for the MPO activity in vivo by different MS methods [48], because it is highly specific, comparably stable, and its concentration correlates with the MPO activity [49–51]. Nevertheless, 3-chlorotyrosine is, first, only a minor product of the protein chlorination by HOCl and, second, it is only an intermediate in biological systems [52]. Since 3-chlorotyrosine reflects only a small percentage of the absolutely released HOCl, its function as a specific MPO activity biomarker is assumed to be limited [53].

The second well studied component in MPO activity studies is taurine (2-aminoethanesulfonic acid), because it is highly abundant in neutrophils with a concentration of about 22 mM [54]. Combined with HOCl, taurine serves as a system to study its fast chlorination by hypochlorite, leading to the stable oxidation product taurine chloramine (Figure 6) that can be conveniently measured via enzyme-linked immunosorbent assay (ELISA) [55]. Taurine chloramine is of low toxicity and reasonable stability and possesses both anti-inflammatory and antimicrobial properties. Its role in acute inflammatory processes is not yet fully understood but

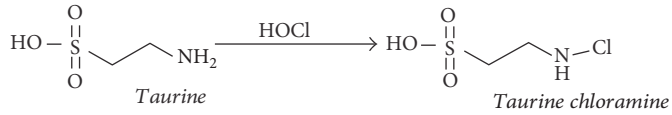


Figure 6: Reaction of taurine, a very important organic (sulfonic) acid which is highly abundant in neutrophils, and HOCl leading to the comparably stable oxidation/chlorination product taurine chloramine, which is used as a marker for the HOCl production rate by MPO and can be easily determined by the taurine chloramine assay. For further details about the assay, see [47].

has been comprehensively discussed by Marcinkiewicz and Kontny [56].

Smaller amounts of HOCl can be determined by using dyes such as 5-thio-2-nitrobenzoic acid (TNB) and 3,3′,5,5′-tetramethylbenzidine (TMB). Using TMB, taurine chloramine is treated with iodide leading to the formation of ICl which is instantly hydrolyzed to hypoiodous acid (HOI). TMB may be used afterwards directly as the chromophore, because it is readily oxidized by HOI to a strongly absorbing blue product [57] that allows the determination of μM quantities of HOCl.

In addition to the TNB and TMB assays, the NAD(P)H assay, the ascorbate assay, and the hydrogen peroxide consumption can also be used to determine the HOCl generation by MPO. These methods were reviewed by Kettle et al. only recently in 2014 [47]. Other potential biomarkers for the MPO/HOCl chlorinating system are either plasmalogens (which will be discussed in more detail later) or glutathione (γ-L-glutamyl-L-cysteinyl-glycin), which result in unsaturated (lyso)PLs and α-chloro aldehydes or glutathione sulfonamide, respectively. These compounds are easily detectable by MS methods which will be shortly discussed at the end of this paper [58, 59].

3. Reactivity of In Vivo Generated HOCl

Due to the fact that the generated HOCl is directly consumed by its reaction with abundant biomolecules, there is increasing evidence that the distribution of HOCl is not homogeneous: MPO is a strongly cationic (positively charged) protein and will therefore be particularly located close to negatively charged compounds, either negatively charged proteins and carbohydrates such as heparin [60], or negatively charged PLs, particularly PS [61], which is almost completely located in the inner leaflet of a membrane, at least in living, non-apoptotic cells. This suggests that MPO binds to apoptotic cells, which leads to an inhomogeneous distribution of HOCl within tissues.

3.1. Generation of Biomolecular Radicals. As it is produced in large quantities and represents a reactive two-electron oxidizing agent in human beings under inflammatory conditions, HOCl is several orders of magnitude more reactive than other oxidants like H_2O_2, (lipid) hydroperoxides, or peroxynitrite [62]. High reactivity normally goes along with nonselectivity. Therefore, HOCl affects not only infiltrated microorganisms, but also "normal" intracellular enzymes, nucleotides, carbohydrates, or lipids in the body with the capacity to change membrane compositions [63]. Nevertheless, HOCl is also a specific oxidizing/halogenating agent that discriminates between different functional groups and reacts normally in the following order: reduced sulfur moieties (thiols and thioethers) > primary and secondary amines > phenols, tertiary amines > double bonds, other aromatics, carbonyls and amides [64]. A survey of calculated and/or experimentally determined second order rate constants (k) are available in [65].

HOCl has the ability to generate free radicals and stable cleavage products by its reaction with small inorganic molecules or functional groups of biomolecules such as proteins, lipids, nucleotides, and carbohydrates. Figure 7 shows some selected examples for the generation of radicals from biomolecules after treatment with HOCl.

3.1.1. Amino Acids and Proteins. Free amino acids can be affected by HOCl in a concentration-dependent reaction at either the sulfur or the amino group in the side chain or the α-amino group of amino acids, the reaction at the sulfhydryl group being the preferred one: previous investigations provided evidence that the amino groups are not affected at all until all sulfhydryl residues have reacted [70]. The chlorination by HOCl at the α-amino group results in transient amino acid chloramines (AACLs), which are known to induce further reactions more selectively than free HOCl. Therefore, AACLs are potential secondary mediators of HOCl-induced injuries [71]. As the most abundant free amino acid (however, not in the strict classical sense, as -COOH is replaced by -SO_3H in taurine), taurine chloramine (Figure 6) is one of the most important products of amino acid chlorination in human PMNs and was already shortly described in the chapter before. In general, AACLs seem to affect biological targets such as cytoplasmic molecules or glutathione and mediate the HOCl-related toxicity leading to apoptotic cell death [72]. Even without any further oxidative reactions, α-amino group AACLs decompose in the presence of water under formation of the corresponding aldehydes [50] or generate, via a one electron pathway, also *N*-centered radicals (Figure 7(a)), which are quickly converted into *C*-centered radicals by different mechanisms, for instance, inter- or intramolecular transport of a proton or decarboxylation [66]. These AACLs decompose to free radicals, which are known to be cytotoxic for tissues and organs [33] and the organism has to try to keep their concentration limited by increased degradation and/or the uptake of antioxidants. It was also shown that HOCl reacts with the same functional groups in proteins as in the case of free amino acids, but these reactions are normally somewhat slower, presumably due to the increased viscosity of protein solutions [73].

Due to their high physiological concentrations in cells, plasma, and most tissues, proteins are actually the most

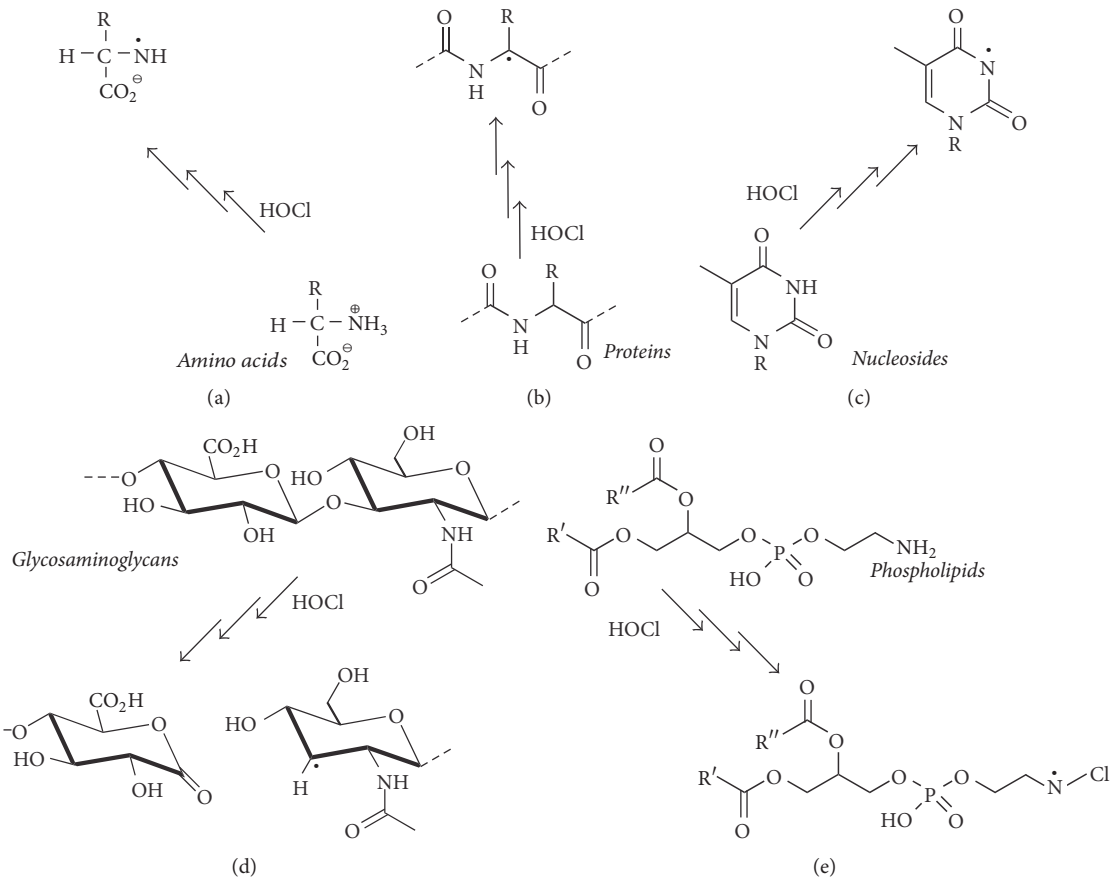


Figure 7: Overview of generated radical species by the reaction of HOCl and different biomolecules. The HOCl-induced chlorination of free amino acids (a, [66]), proteins (b, [66]), nucleosides (c, [67]), glycosaminoglycans (d, [68]), and PLs (e, [69]) leads in various consecutive reactions (implied by three arrows) to different radical species which may cause oxidative stress and favor the development of many diseases.

relevant targets for in vivo modification by HOCl and other oxidants causing numerous posttranslational modifications such as the oxidation of sulfhydryl groups and the chlorination of aromatic side chains or the chlorination of amino groups in the side chains and some other side reactions [42]. It is assumed that HOCl primarily affects more readily the functional groups in the side chains of proteins than in the peptide linkages [74]. The side chain chlorination comprises aggregation, oligomerization, destabilization, fragmentation, and/or degradation steps [42] after the formation of an *N*-centered radical which is further converted to a *C*-centered radical (Figure 7(b)) often catalyzed by transition metal ions (e.g., Cu^+) [66]. Cysteine and methionine (sulfur-containing amino acids) are characterized by an about 100-fold higher reactivity with HOCl than any other functional group of proteins or other biomolecules. The HOCl-initiated chlorination of cysteine leads to labile sulfenyl chloride (R-S-Cl) and sulfenic acid (R-S-OH) intermediates, which are both known to undergo further oxidation steps with HOCl [75]. These irreversible modifications result in sulfinic ($R\text{-}SO_2H$) or sulfonic ($R\text{-}SO_3H$) acids as well as thiosulfinate products ($R\text{-}S\text{-}SO\text{-}R_2$), the formation of which is accompanied by protein decomposition [5]. Methionine is almost as readily affected as cysteine and may either generate (under in vivo conditions) the reversible methionine sulfoxide or the irreversible dehydromethionine [76]. Beside sulfur-containing amino acids, the amino groups in lysine, histidine, and arginine are also preferred targets for HOCl. Although the reaction rates are reduced in comparison to cysteine, the majority of reaction products are generated irreversibly in vivo and are thus prone to further reactions with other potential reactive protein sites and/or other biomolecules [74].

3.1.2. Nucleic Acids and DNA. The HOCl-induced oxidation of nucleic acids in a DNA strand normally affects the nitrogenous base either at the primary nitrogen ("exocyclic") in adenosine, guanosine, or cytosine or at the secondary nitrogen ("endocyclic") in guanosine, uridine, or thymidine, the reaction at the endocyclic nitrogen seemingly being preferred [11]. These *N*-chlorinated products are intermediates and subsequently converted into more stable chlorinated products such as 5-chlorocytosine, 5-chlorouracil, 5-chloro-(2′-deoxy)-cytidine, 8-chloroadenine, 8-chloro-(2′-deoxy)adenosine, and 8-chloro-(2′-deoxy)guanosine [77, 78]. Using electron paramagnetic resonance (EPR) in combination with spin trapping to enhance the lifetime of the radicals of interest, the generation of *N*-centered radicals of nucleosides (Figure 7(c)) from transient chloramines could

be monitored by disrupting the double-stranded DNA [67]. The tendency of radical formation of isolated nucleosides as well as polynucleotides can be sorted as follows: cytosine > adenosine > guanosine > thymidine [67, 79]. Some of these products were determined both in inflammation-related cell cultures and in inflamed tissues and are known to cause the dissociation of the double-stranded DNA (cleavage of the hydrogen bonds) and are therefore potential biomarkers of early inflammatory processes [80]. It is known that the concentrations of chlorinated nucleosides (5-chlorouracil) are elevated in the inflammatory exudate of humans and rats as well as in atherosclerotic plaques (AP) [55]. Moreover, there is evidence for the cytotoxic and mutagenic potential of chlorinated nucleic acids or chlorinated DNA moieties (e.g., [77, 81–84]). These cytotoxic effects (i.e., frameshift mutations or base pair substitutions) can be caused by both lipid peroxidation products and HOCl-mediated protein-DNA crosslinks. Under neuropathological conditions, HOCl is continuously released from PMNs or microglia to diffuse into the brain parenchyma and maintains chlorinative stress resulting in neurodegeneration [44]. Even if noncovalent ionic interactions of DNA and proteins are physiologically very important for normal cellular functions [85], the oxidation of covalently bound protein-DNA cross linkages leads to inadequate DNA repair [86] and to mutagenic and cytotoxic effects of phagocytes on microbial pathogens and host tissue [87].

3.1.3. Carbohydrates. Oxidized carbohydrates were investigated to a much lesser extent, presumably due to their complexity [88]. As already discussed in the context of other biomolecules, free amino groups in sugars such as glucosamine are the preferred targets for HOCl. Although the reaction between HOCl and common monosaccharides is comparably slow [89, 90], the HOCl-induced transformation of free amino groups in sugars was reported to be even faster than the modification of the *N*-terminal amino group of peptides [91]. Moreover, HOCl causes the generation of chloramides (R-NCl-C(O)-R_2) from amide groups (R-NH-C(O)-R_2) of glycosaminoglycans (GAGs) in the extracellular matrix [92]. Since hyaluronan and chondroitin sulfate regulate different cellular and tissue functions and possess an *N*-acetyl group in the repeating disaccharide unit, they are the most intensely investigated GAGs in the extracellular matrix [93], another reason why GAGs are intensively studied is their abundance in cartilage (chondroitin sulfate) or synovial fluid (hyaluronan), where inflammation plays a major role [88]. Due to the poor oxidizing properties of the initially generated chloramides, they are prone to reacting with different reducing agents including radicals [92] and are thus only transient products. Chloramides decompose to *N*-centered radicals (R-N$^{\bullet}$-C(O)-R_2) followed by *C*-centered radicals, leading finally to the site-specific fragmentation of the glycosidic linkages in the polysaccharide chain (Figure 7(d)) under generation of GAG oligosaccharides [68]. These reactions are stimulated by the presence of low-valent transition metals such as Fe^{2+} or Cu^{+} via a one-electron reduction of the chloramide group [68]. Since GAGs are known to be involved in important cellular functions, it is assumed that oxidatively modified GAGs may lead to altered cell migration, adhesion, proliferation, growth, and even phenotyping [33]. Finally, it is known that high mass hyaluronan has anti-inflammatory properties, while low mass hyaluronan is proinflammatory [94]. Therefore, changes of the molecular weight of hyaluronan are of major importance.

3.1.4. Phospholipids. The last important class of biomolecules that can be affected by HOCl is represented by lipids, particularly PLs. It is well known that HOCl reacts with either the double bonds of the acyl chains of unsaturated PLs or the reactive functional groups in the headgroup (e.g., the amino residues in PE and PS). Although the focus of this review is on the addition of HOCl as a nonradical (molecular) agent to the double bonds of PLs which leads to chlorohydrin generation, HOCl can also trigger the formation of *N*-centered radicals in PE and PS by reaction with the nitrogen in the headgroup. The first step of the amino modification by HOCl is the generation of monochloramine, which can be converted (in the presence of a sufficient excess of HOCl) into the corresponding dichloramine [95], which are both only fairly stable compounds. Using EPR with spin trapping, Kawai et al. could monitor the generation of an *N*-centered radical from the PE dichloramine (Figure 7(e)) [69]. Via direct or radical-mediated processes, the elimination of HCl may also occur and result in the generation of the corresponding nitrile (Equation (3)), while an aldehyde is the final product of the hydrolysis of the monochloramine (vide supra)

$$\text{R-CH}_2\text{-NCl}_2 \longrightarrow \text{R-CN} + 2\text{HCl} \quad (3)$$

We will not focus on these aspects here to a major extent. The reader who is particularly interested in the related reaction mechanisms and further details about free radical formation from biologically important molecules by HOCl is referred to the excellent review by Panasenko and coworkers [33]. However, it is widely accepted that the radicals derived from chloramines may induce or mediate (through, e.g., tocopherol) oxidative modifications of lipids [96].

4. Phospholipid Oxidation by HOCl

A crude survey of products which may be expected if PLs are subjected to HOCl oxidation is shown in Figure 8 [97].

The most relevant PL oxidation products can be sorted into (i) full chain length products (hydroperoxides, hydroxides, and epoxides), (ii) full-chain rearranged products (isoprostanes and isofurans), and (iii) truncated products (alkanals, alkenals, and hydroxyalkenals) [99]. Although compound class (iii) is currently most intensely investigated, because aldehydes may react (under the particular generation of Schiff bases and other products) with proteins and modulate in this way the activity of enzymes [100], the compounds we will discuss here in more detail belong to group (i): no truncation of the fatty acyl residue occurs if HOCl reacts with the double bonds of PLs or FFAs under generation of chlorohydrins as addition products.

The reaction between olefinic compounds (such as unsaturated lipids) and HOCl is known for more than a hundred years, including discussions about which compounds can be

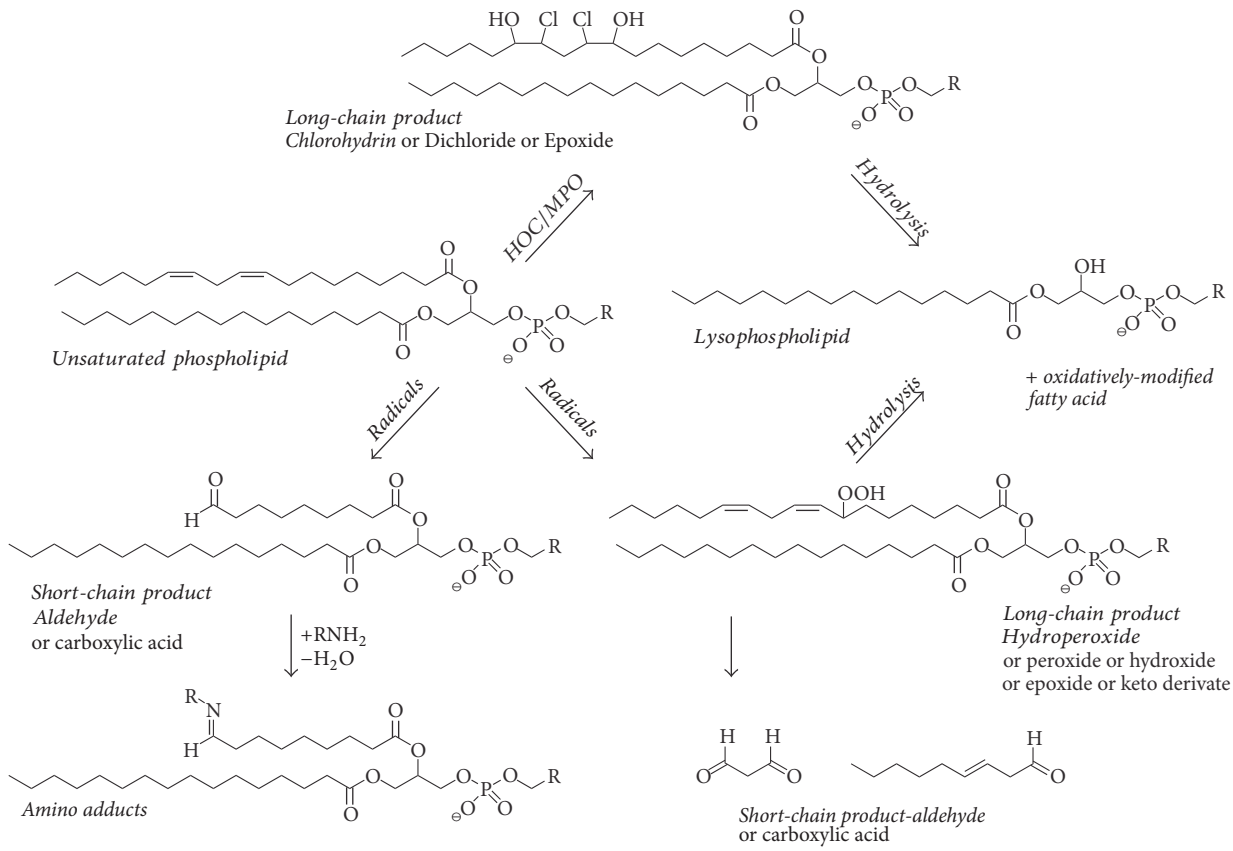


Figure 8: Scheme of potential reaction products from an unsaturated PL under inflammatory conditions in vivo. Please note that the term "long-chain" products refers to compounds that have a higher mass in comparison to the native PL while "short-chain" products have a smaller mass. A similar scheme has been suggested recently [97]. Although lysophospholipids (LPLs) might be also considered as "short-chain" products, they are discussed here as a distinct lipid class. This scheme should be regarded as a very crude survey and not all compounds that play a physiological role are actually listed (reprinted from [98] with permission from Elsevier).

considered as the stable final products: chlorohydrins, glycols [101], or epoxides [102]. Nowadays, it is commonly accepted that chlorohydrins (the addition products of HOCl to the double bond) are the most abundant products when lipids are treated with HOCl, even if there are also recent reports that dimeric products of oleic acid may be also generated [103].

Beside PLs (as shown in Figure 8) containing two fatty acyl residues, which are linked to the glycerol by ester linkages, alkenyl-acyl PLs, commonly termed "plasmalogens," are a second class of PLs that occur in significant amounts in many biological samples, for instance, in neutrophils, macrophages, glia cells, neurons, smooth muscle cells, endothelial cells, cardiac myocytes, and particularly spermatozoa [104, 105]. These plasmalogens are generated by the reaction between glycerol and a fatty aldehyde followed by the elimination of one water molecule and are often characterized by a choline or (even more abundant) an ethanolamine headgroup [106]. Although brain and spermatozoa lipids contain considerable amounts of highly unsaturated fatty acyl residues (such as docosahexaenoic acid, 22 : 6) [107], it is nowadays commonly accepted that the alkenyl ether linkage represents a site of high reactivity with a large variety of ROS [108], much higher in comparison to the "normal" double bonds along a common fatty acyl chain [109]. Therefore, plasmalogens are often considered to represent important biological antioxidants [110]. It is important to note that the resulting plasmalogen LPCs possess an unsaturated fatty acyl residue in the *sn*-1 position [111], which is a remarkable difference in comparison to diacyl-PCs that possess the unsaturated fatty acyl residue normally in the *sn*-2 position and yields exclusively saturated LPCs upon oxidation with HOCl. Therefore, the detection of unsaturated lysophospholipids (LPLs) is normally a clear indication of the presence of plasmalogens [112].

5. Beneficial Effects of Lipid Chlorohydrins and Peroxidized Lipids

The majority of authors indicate that lipid oxidation processes and the related products exert a negative effect on the organism and that everyone should attempt to minimize the extent of lipid oxidation, for instance, by the ingestion of antioxidative compounds such as vitamins [113]. However, there are also a few indications that lipid oxidation positively affects the organism [13].

There are indications that oxidized lipids are faster digested by phospholipases (particularly phospholipase A_2 (PLA_2), which cleaves exclusively the fatty acyl residue in *sn*-2 position and plays a more prominent role than PLA_1) than their native (nonoxidized) counterparts. This emphasizes that the organism recognizes oxidized lipids as "foreign" and is thus interested to get rid of these molecules as fast as possible. This increasingly important aspect has been reviewed recently [114]. Conversely, this means that PLA_2 enzymes (there are many different species) affect oxidized PL more readily: for instance, it could be shown that AP are characterized by an elevated content of lysophosphatidylcholine (LPC), which is presumably generated by the action of PLA_2 on PC [115]. Although there are many indications that peroxidized lipids are preferentially digested by PLA_2, the detailed mechanisms behind this finding are not yet completely clear. The two potential reasons are, first, the activity of the enzyme might be actually elevated in the presence of a different substrate, because this substrate fits more readily into the active site of the enzyme, and second, the presence of oxidized lipids may also be accompanied by alterations of the lipid membrane structure and this might lead to improved digestibility [116].

Different effects in comparison to peroxidized lipids have been described for HOCl-modified lipids: halogenated PLs are inhibitory to the enzyme and elevated concentrations of chlorinated (and brominated) PC molecules decrease the activity of secretory PLA_2 by about 50% [34]. This could be confirmed recently by using bee venom PLA_2 and liposomes of defined lipid compositions [148]. These notable differences clearly emphasize that more detailed studies of these aspects are necessary.

In general, it is known that oxidized lipids (especially oxidized arachidonyl residues) can induce protective effects by the upregulation of antioxidant genes, inhibition of inflammatory signaling pathways through Nrf2 (nuclear factor erythroid 2-related factor 2) mechanisms [149], antagonism of Toll-like receptors [150], immunomodulating and immunosuppressive action of oxidized PLs in adaptive immunity and autoimmune disease [13], activation of PPARs (peroxisome proliferator-activated receptors) which are known for their anti-inflammatory action, and the protective action against lung edema in acute lung inflammation [151]. As implied before, plasmalogens are also known as antioxidants.

6. In Vitro Generation of Chlorohydrins (Cell and Tissue Studies)

Although many papers focus on the effects of (per)oxidized lipids on selected cell lines, the effects of chlorohydrins were studied to a much lesser extent. This might be due to the fact that PL chlorohydrins (unlike, e.g., "oxPAPC" [152]) are nowadays not commercially available. However, this purchasable "oxPAPC," which is obtained by air oxidation of PAPC, is a relatively crude mixture and contains at least three different substance classes (hydroperoxides, aldehydes, and carboxylic acids). This makes detailed assignments about which compound is responsible for the observed effects very difficult.

In one of the first chlorohydrin-related studies, Carr and coworkers treated human erythrocytes with HOCl [153] and were able to detect the generation of chlorohydrins by means of thin-layer chromatography (TLC) and by using an ELISA assay against chlorohydrins. Since lipid chlorohydrins are more polar and bulky than their parent lipids [154], their impact on membrane stability and integrity was also investigated by measuring the release of hemoglobin from the cells. Different chlorohydrins caused different effects: the addition of HOCl-treated oleic acid to the red blood cells resulted in a rapid (concentration-dependent) lysis of the cells while the effect of cholesterol CH was less pronounced and was also dependent on the type of the CH isomer with the chlorohydrin-3 being the most deleterious one. Further analysis by Vissers and coworkers [155] examining HOCl-lysed red cell ghosts by electron microscopy demonstrated considerable disruption of the cell membrane and provided evidence for complete rupture at an HOCl concentration of about 5 mM.

The same authors [156] extended their work a few years later to another cell system: when human umbilical vein endothelial cells were treated with preformed halohydrins of oleic acid, cell detachment and necrotic death can be induced with increasing doses of the halohydrins, whereas the cells were unaffected by equivalent doses of pure oleic acid as the control. Bromohydrins (which can be obtained by the treatment of lipids with HOBr which can be conveniently generated by mixing HOCl with a small excess of NaBr) caused even more lysis than the corresponding chlorohydrins at equivalent doses, presumably because bromohydrins are more readily incorporated into the endothelial cells. It was also suggested that membrane protein modification is the reaction which is primarily responsible for HOCl-mediated cell lysis [157]. Therefore, further studies of these aspects are required.

It is important to note that chlorohydrins seem to have not only structural relevance but are also actively involved in metabolic pathways. It could be shown [158] that the cellular adenosine triphosphate (ATP) level is significantly diminished when cultured myeloid (HL60) cells are incubated for 24 h with selected chlorohydrins. These effects were additionally compared with the effects of different small aldehydes (such as 4-hydroxy-2-nonenal, HNE) which are known to be generated under inflammatory conditions by cleavage at the double bond position of unsaturated fatty acyl residues. The ATP depletion by the PL chlorohydrins was slightly less than that of HNE, but greater than that of hexanal and trans-2-nonenal, which are all well-known oxidation products of lipids. This indicates that chlorohydrins directly affect the activities of enzymes.

Robaszkiewicz and coworkers [159] studied the effects of chlorohydrin PCs on human erythrocytes. It could be shown that the biophysical properties of PL bilayers of known compositions are altered in the presence of PC chlorohydrins. In particular, changes in erythrocyte shape (echinocyte formation) and aggregation were significantly altered when vesicles containing PC-CH were mixed with the cells. Similar effects were also found [160] when HUVEC-ST (endothelial cells) were treated with PC chlorohydrins. Under these conditions

there was a decrease of the mitochondrial potential and an increase in the number of apoptotic cells. These effects were accompanied by an increase in the level of active caspase-3 and caspase-7 and a decrease in glutathione content and the overall antioxidant capacity of the cells. Similar effects were observed in the case of lung epithelial cells and HOCl was found to affect the redox state of A549 cells by oxidation of GSH, inactivation of antioxidant enzymes, and the increase of ROS generation [161].

It could also be shown that induced acute pancreatitis results in a substantial release not only of FFAs but also of the chlorohydrins of both oleic and linoleic acid from adipose tissue while accompanying plasma investigations evidenced only the chlorohydrin of oleic acid. Administration of 250 μM lipid chlorohydrins, the concentration found in ascitic fluid, induces the expression of TNF-α and IL-1β in peritoneal macrophages and increases the systemic inflammatory response in pancreatitis. Finally, increased concentrations of oleic acid chlorohydrin have been found in the plasma of human patients with pancreatitis [162].

7. Chlorohydrins in Diseases

The last example indicates that there is increasing evidence that products derived from the MPO/H_2O_2/Cl^- system play a crucial role in many diseases and it can be suspected that this neutrophil-derived system is, beside inflammation mediated by T-cells, a major contributor in inflammatory diseases [163, 164]. A survey of different diseases mediated by MPO activity is given in Table 2, showing only the most important diseases due to the limited space available.

7.1. Atherosclerosis. There is increasing evidence that many diseases are accompanied by inflammation. This does not only apply for classical inflammatory diseases such as gastritis and arthritis, but also for diseases such as obesity and cancer [165]. The most important and therefore most intensively studied disease is atherosclerosis [166], which is characterized by the thickening, hardening, and loss of elasticity of the walls of arteries up to the buildup of fatty plaques in the artery walls. One probable mechanism is mediated by the so-called foam cells described by the uncontrolled uptake of cholesterol and other lipids by macrophages which is presumably triggered by oxidized lipids [167].

The first hint to the involvement of chlorinated lipid species in atherosclerosis was obtained in 1996 [168]: Hazen and coworkers detected different chlorinated lipids upon the exposition of human LDL to the complete MPO system at acidic conditions with the focus on cholesterol: beside dichlorinated cholesterol, cholesterol α-chlorohydrin (6β-chlorocholestane-(3β,5α)-diol), cholesterol β-chlorohydrin (5α-chlorocholestane-(3β, 6β)-diol), and a structurally related cholesterol chlorohydrin could be detected and characterized by MS. The authors provided additional evidence that Cl_2 derived from HOCl (particularly at acidic conditions which favor the presence of the free acid HOCl) is the actual chlorinating intermediate in the oxidation of cholesterol by MPO. It was suggested that Cl_2 generation in acidic compartments constitutes one important pathway for the oxidation of LDL cholesterol in the artery wall.

A few years later, the presence of lipid chlorohydrins could be unequivocally confirmed in atherosclerotic lesions and it was also found that chlorinated lipids exhibit serious effects on different cell lines which may contribute to the development of the disease. These findings suggest that PL chlorohydrins formed in HOCl-treated LDL could contribute to the proinflammatory effects observed for this modified lipoprotein in vitro [169]. An interesting experiment was performed by Yang and coworkers in 2006 [170]: they infused clamped carotid arteries with 1 mM HOCl for 1 h, before reperfusion and animal recovery for up to 2 weeks. Interestingly, the results of these studies indicate that the addition of HOCl alone can lead to neointima formation containing both VSMCs (vascular smooth muscle cell) and macrophage infiltration that is consistent with the process of atherosclerosis.

A surprising observation was made in 2008 [171]. Although it is commonly accepted that the LPC levels are elevated in sera from patients with atherosclerosis and in atherosclerotic tissues [172], these are regularly saturated LPC species, as the majority of biologically relevant PLs contain an unsaturated fatty acyl residue in the *sn*-2 position and LPCs are typically generated by the enzyme PLA_2 which cleaves the fatty acid in the *sn*-2 position. The authors demonstrated for the first time that LPC-chlorohydrins are elevated over 60-fold in human atherosclerotic lesions and therefore may have unique proatherogenic properties compared to common saturated LPCs. This emphasizes the role of plasmalogens in the disease [110]. The important role of plasmalogens in AP could already be shown in 2003 by Thukkani and coworkers as they found a 1400-fold higher concentration of the α-chloro fatty aldehyde (α-ClFALD) 2-ClHDA compared to normal aorta samples [173]. Interested readers will find more information about the role of plasmalogens in atherosclerosis in a recent review by Ford [174]. It has also been hypothesized recently that the type of the generated LPC is of (patho-)physiological relevance [175], because LPC is a major constituent of oxidatively modified LDL and is generally considered to be atherogenic. However, some studies have also shown antiatherogenic properties of some LPC species [175]. These controversial findings are apparently caused by changes of the degree of saturation of the fatty acyl moiety of the LPC species. The presence of (ω)-PUFAs at the *sn*-1 position of LPC makes LPC anti-atherogenic [176], while the presence of saturated fatty acids renders LPC atherogenic [177]. It is important to note that LPC is not only generated by PLA_2, but LPC may be considered as a general product of the decay of oxidized lipids. This aspect will be discussed in more detail below.

7.2. Arthritis. Upon acute inflammation, the synovial fluid from patients suffering from rheumatic diseases usually contains a large number of granulocytes and cell numbers of the order of about 1×10^9 cells in 30 mL synovial fluid are not exceptional [178, 179]. Since neutrophils (beside macrophages) are a rich source of the enzyme MPO, it is not surprising that synovial fluids are also characterized by

significant MPO activities [120]. It is well known from investigations using classical assays such as the determination of thiobarbituric acid reactive substances, lipid hydroperoxides, or diene conjugates that the lipid compositions of synovial fluids change significantly under inflammatory conditions [180]. Although little attempts were performed to characterize the entire profile of oxidized lipids in the synovial fluids [181], it could be shown in 2005 that the LPC content correlates with the severity (inflammatory state) of the disease and even the success of a medical cure could be monitored by the decreasing LPC content [182]. In 2015 novel peptidoaldehydes from GSH and α-ClFALD in human neutrophils and a mouse model could be identified for the first time, indicating that α-ClFALD is produced as a result of MPO activity [183]. There is also increasing evidence that LPC represents just a transient compound [184]: lysophosphatidic acid (LPA) is mainly produced by the hydrolysis of LPC catalyzed by the enzyme lysophospholipase D, which is also called autotaxin (ATX). LPA interacts with specific G-protein coupled receptors and is involved in the regulation of cellular survival, proliferation, differentiation, and motility. Therefore, an elevated LPC content (mediated either by ROS or PLA_2 activity) may be considered an important prerequisite of LPA generation. The inhibition of the LPA receptor has also been suggested as a promising way to suppress the symptoms of arthritis [185].

7.3. Other Diseases. There are many other diseases, for instance, multiple sclerosis, pulmonary fibrosis, liver fibrosis, and hepatitis, which are characterized by elevated LPC contents and/or enhanced MPO activities and/or enhanced lipid peroxidation product contents. These diseases have recently been reviewed and the interested reader is advised to consult [186].

8. Analysis of Chlorinated Lipids Using Different Mass Spectrometry Methods

Lipid oxidation products are classically assessed by photometric assays such as the determination of the thiobarbituric acid reactive substances (TBARS), the diene conjugates, or the number of hydroperoxides. Although these assays are simple and sensitive [187], they are not the methods of choice to determine chlorinated lipids. MS techniques using different ionization methods are much better suited for that purpose and will therefore be shortly discussed in this chapter. The HOCl-induced oxidation of plasmalogens was also investigated by MS, where plasmalogens gave even higher yields of LPC than diacyl-PCs with the same number of double bonds [109], confirming the extreme oxidation-sensitivity of plasmalogens. Since the analysis of FFA is easier in comparison to (phospho)lipids, they were formerly nearly exclusively investigated focusing on gas chromatography mass spectrometry (GC-MS) methods.

8.1. Gas Chromatography Mass Spectrometry. By using GC combined with different detection methods (i.e., ultraviolet (UV), MS, flame ionization detector (FID), or fluorescence) FFA oxidation products can be easily studied and the identification of many different products is made possible. Although GC offers an excellent resolution (even the separation of isomeric FFA is possible), only volatile organic compounds are detectable, which requires a mandatory esterification step of the FFA (often the fatty acid methyl ester, FAME) [188]. For more detailed information about sample preparation and GC-MS procedure regarding FFA analysis see also [189]. Using GC-MS, increased concentrations of fatty acid chlorohydrins were identified in adipose tissue, which emphasizes the systemic inflammatory response in acute inflammation [162]. Nevertheless, it has to be stressed that even the oxidation chemistry of simple compounds is very complex and increases with the complexity (number of double bonds) of the compound of interest. For instance, even the oxidation of isolated linoleic acid yields dozens of different products and this sheds light on the complexity of the product pattern [190].

Since PLs are nonvolatile, their direct separation and analysis with GC-MS is not possible. Therefore, the analysis of the fatty acyl composition of PL species requires a time-consuming sample preparation including the PL hydrolysis into diacylglycerols and FFA followed by their conversion into FAMEs [188]. However, there are some applications for the analysis of oxidized and chlorinated lipids using GC-MS. Due to their considerable reactivity with HOCl, plasmalogens readily generate oxidation products (i.e., α-ClFALD) and LPLs that are suitable for GC-MS analysis. α-ClFALDs are both hard to ionize and relatively unstable under ESI conditions. Thus, the conversion with pentafluorobenzyl (PFB) hydroxylamine into oximes improves the detection sensitivity by GC-MS, which is better in comparison to the achievable sensitivity of ESI MS [191]. In this study 2-chlorohexadecanal was investigated by GC negative ion chemical ionization (NICI) MS after PFB hydroxylamine derivatization of tissue and cell culture samples as an alternative method to LC-MS.

8.2. Electron Ionization Mass Spectrometry. Significant progress could be made in the last decades regarding the MS analysis of nonvolatile and/or high mass products. Until the eighties of the last century conventional electron ionization (EI) MS was nearly exclusively available. Using this approach, the sample is evaporated and subsequently ionized in the gas phase by collision with accelerated electrons, which (normally) leads to the removal of one electron from the analyte and the generation of radical cations. The most important prerequisite is sufficient volatility of the analyte. Therefore, this method can hardly be applied to lipids but is applicable to fatty acids subsequent to their conversion into methyl or trimethylsilyl esters to enhance their volatility [192].

Although the application range of EI is limited, the need to use volatile compounds enables the direct and straightforward combination of EI MS with GC. Among all chromatographic methods, GC offers the best separation quality for FFA and enables even the separation of different isomers. For instance, Winterbourn and coworkers [193] were able to show that both 9,10-chlorohydrin isomers of oleic acid can be differentiated by GC and that both isomers are generated in the same amount if oleic acid reacts with HOCl. Therefore, it can be concluded that the reaction

between HOCl and unsaturated fatty acids is not regiospecific at all but results in a mixture of isomers. These authors have also suggested that the incorporation of chlorohydrins into cellular membranes leads to the destabilization of the lipid membranes, chlorohydrins, thus representing potential biomarkers. Similar data could be obtained by the same group regarding the chlorohydrins of cholesterol [194]. However, the biological significance of lipid chlorohydrins so far remains to be elucidated in more detail and there is also increasing evidence that the reaction between FFAs such as oleic acid and HOCl is even more complex than initially assumed. Recently, Schröter and colleagues [103] found that in addition to chlorohydrin as the main product of the reaction between HOCl and oleic acid dimeric and trimeric products are generated, the abundance decreasing from dimer to trimer. These oligomeric products were monitored by using high performance TLC (HPTLC), electrospray ionization (ESI), and matrix-assisted laser desorption and ionization (MALDI) MS. These oligomeric products might be derived either from the contribution of free radicals or from estolide formation [195].

8.3. Soft Ionization MS. Since the 1980s, a continuous stream of very creative papers was published in which new "soft ionization" techniques were presented enabling the MS analysis of native and oxidatively modified lipids without the need of previous saponification and derivatization. In contrast to EI, soft ionization MS enables direct detection of the molecular ion of the analyte. Typical soft ionization methods are ESI, APCI, and MALDI that enable direct analysis of native lipids and the corresponding oxidation products without the need of sample derivatization (like in GC-MS methods). In contrast to EI, the sample is not ionized by the loss of an electron but by the addition of a cation (often H^+ or Na^+, which are both omnipresent in biological samples) or deprotonation. Radical cations are normally not observed.

Basically, ESI and APCI are "softer" methods and can also easily be coupled to chromatographic separation methods such as High Performance Liquid Chromatography (HPLC). By contrast, MALDI is the more convenient method which tolerates a significant extent of impurities. Also, it generates almost exclusively singly charged ions, which makes MALDI spectra interpretation much easier [196].

8.3.1. MALDI-TOF MS. As early as in 2001, Arnhold and coworkers [197] were able to show that MALDI-TOF MS is a convenient method to study the effect of the reagent HOCl as well as the complete $MPO/H_2O_2/Cl^-$ system on selected PCs with differently unsaturated fatty acyl residues. It could be shown by using polyunsaturated lipids (with arachidonoyl or docosahexaenoyl residues) that chlorohydrins and glycols are the most relevant products. The corresponding yields of these products could be influenced by varying the incubation time: monochlorohydrins and glycols dominated at short incubation times, while bischlorohydrins as well as products containing one chlorohydrin and one glycol moiety appeared after longer incubation. There was also evidence that LPC (lacking one fatty acyl residue in comparison to the native PL) is another important reaction product. This aspect was investigated one year later in more detail [198]. It turned out that the yield of LPC increases if PCs with a higher content of double bonds are oxidized by HOCl. This is due to the fact that PCs including an oleoyl residue (18 : 1) give only a small amount of LPC, while PLs with a docosahexaenoyl residue (22 : 6) result in considerable amounts of LPC. The generation of LPC under the influence of HOCl was explained by the introduction of electronegative elements (chlorine and oxygen) that withdraw electrons from the ester bond, thus enhancing its sensitivity to hydrolysis [198]. This is exemplarily shown in Figure 9. It is particularly notable that LPC may also be generated when phospholipases are completely absent.

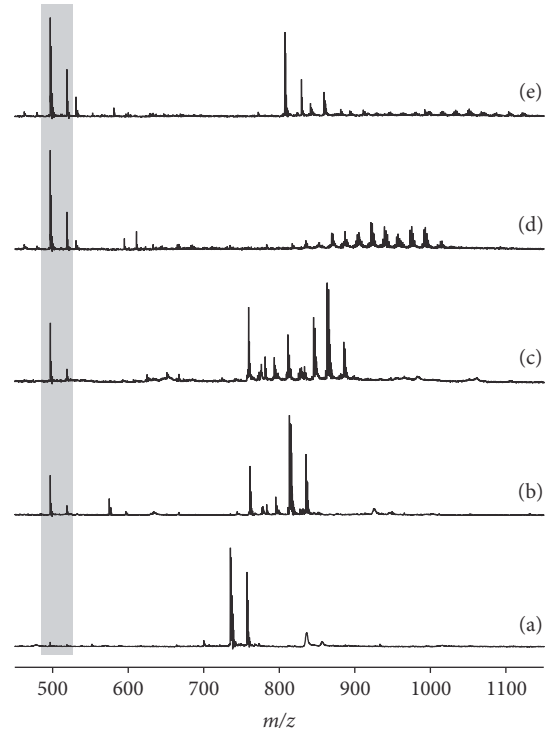


Figure 9: Formation of LPLs and higher molecular weight products (particularly chlorohydrins) after incubation of different PCs with an excess of HOCl as evidenced by positive ion matrix-assisted laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF MS): (a) PC 16 : 0/16 : 0; (b) PC 16 : 0/18 : 1; (c) PC 16 : 0/18 : 2; (d) PC 16 : 0/20 : 4; (e) PC 16 : 0/22 : 6. Please note that the yield of lysophosphatidylcholine (LPC, marked by the grey box) increases with the extent of unsaturation of the used PC (reprinted with slight modifications from Arnhold et al. [198] with permission from Elsevier).

Although this mechanism should be valid for all unsaturated lipids independent of the headgroup, the extent of LPL generation depends significantly on the structure of the used lipid: it was shown recently that the HOCl treatment of PE does not lead to LPE generation [199]. However, there is so far no convincing explanation for this surprising difference, and further studies to clarify this problem are urgently required.

In contrast to HOCl-induced lipid oxidations with LPC as the main product, hydroxyl radicals primarily cause scission at the position of the double bonds under generation of the corresponding aldehydes and/or carboxylic acids. This is a notable difference, although the detailed molecular reasons are still unknown. Finally, it has to be emphasized that a differentiation between HOCl and hydroxyl radicals under physiological conditions is still difficult: if an unsaturated PC is treated with the Fenton reagent in the presence of NaCl, the generation of chlorohydrins can be unequivocally monitored [200]. This implies that the in situ generated hydroxyl radicals react with the chloride under formation of HOCl. This might also be the reason why water radiolysis (the best characterized method of $HO^{\bullet}$ generation) may also lead to the generation of HOCl if water radiation is performed in the presence of an elevated salt content [201]. This can be easily verified in cell experiment by measuring the number of dead cells in dependence of the salt concentration at a constant γ-dose [202].

Finally, a warning is required: although MALDI is a soft ionization method, it is less soft than ESI MS. While chloramines of PE can be easily detected by ESI MS [95], this is not possible by MALDI MS, at least if standard MALDI matrices but not the optimum matrix is used [203]: 4-chloro-α-cyanocinnamic acid is the MALDI matrix of choice for the detection of chloramines. Although not directly within the scope of this topic, the detection of peroxidized lipids by MALDI MS is also difficult. However, there are indications that their detection can be improved if the analytes are desorbed from a target coated with nitrocellulose [204].

8.3.2. Native Lipid Mixture Analysis by ESI MS. As already indicated, ESI MS can be easily combined with chromatographic separation. Therefore, ESI coupled to HPLC (or more recently nano-HPLC) is normally the method of choice if complex lipid mixtures have to be analyzed [205]. This particularly applies because lipid oxidation products are normally only present in very small amounts in biological systems, while the native bulk lipids are by far more abundant [99]. Compounds which are present in small amounts only are normally suppressed by more abundant compounds and are thus often not detectable if the entire lipid mixtures are investigated without previous separation. The same applies if lipids with different headgroups and different tendencies to form a positive charge are investigated [206].

In a pioneering work Jerlich and coworkers [207] have shown that combined LC-ESI MS is a suitable method to monitor the oxidation products if PCs in human LDL are treated with either HOCl or the complete MPO system. It could be shown that chlorohydrin products from lipids containing oleic, linoleic, and arachidonic acids can be easily detected, but no hydroperoxides of linoleoyl or arachidonoyl lipids could be monitored. This is an important finding, since hydroperoxides were previously suspected to represent an additional reaction product which undergoes free radical-mediated reactions in the presence of HOCl [208].

Somewhat later the same authors [209] were able to show that there is a direct correlation between the content of oxidation products in the LDL sample on the one hand and the concentration of HOCl, or the MPO activity as well as the acidity of the medium, on the other hand. This could be also verified by the exposition of defined PC species to stimulated neutrophils [210]. It is nowadays commonly accepted that LC-MS is the method of choice to analyze lipid chlorohydrins, which has been reviewed recently [191]. Nevertheless, the limited commercial availability of oxidized PL standards aggravates the method development enormously. Compared to chlorinated cleavage products (α-ClFALD) of plasmalogens, their LPL chlorohydrins are easily detectable with ESI MS even without previous HPLC purification/separation [211]. These LPC-ClOH were detected by MS/MS using for example, the neutral loss scan (i.e., m/z 95 for trimethylamine of the phosphorylcholine headgroup) [212].

8.3.3. Native Lipid Mixture Analysis by APCI MS. Atmospheric pressure chemical ionization is a useful method which has facilitated lipid analysis for many years. Many classes of lipids including FFAs, PLs, sterols, and triacylglycerols are ionizable by APCI [213]. Although APCI is a soft ionization method, the relatively harsh conditions employed in the source induce some degree of fragmentation. This is sometimes helpful if no MS/MS capacity is available but aggravates the analysis of complex mixtures. Another problem regarding quantitative analysis is the strong impact of the extent of unsaturation on the ion yield. TAG can be conveniently analyzed by APCI, but APCI is not the method of choice to analyze PLs due to the relatively low sensitivity achievable. To the best of our knowledge there are only very few reports where APCI was used to analyze oxidized glycerolipids [214]. However, APCI is an excellent technique to analyze oxidized fatty acids [215]. Surprisingly, chlorinated lipids have so far not been analyzed by APCI.

8.4. Other Methods to Monitor Chlorohydrin Generation. There are not many methods [216] for evaluating the generation of lipid chlorohydrins apart from MS, but at least a few of them should be shortly mentioned.

8.4.1. Nuclear Magnetic Resonance. Although Nuclear Magnetic Resonance (NMR) suffers from comparably low sensitivity, it has often been used to study the reaction between HOCl and unsaturated fatty acids or even PLs. ^{1}H NMR was used in the majority of cases [217] since ^{1}H is the most sensitive NMR nucleus. The progress of the reaction between the unsaturated lipid and HOCl can be easily monitored by the disappearance of olefinic residues and the formation of the typical chlorohydrin resonances that can be easily differentiated from the resonances of simultaneously generated glycols and epoxides [89]. Due to the limited sensitivity of ^{13}C NMR there are no reports of ^{13}C NMR studies of lipid chlorohydrins. However, there were a few attempts to use ^{31}P NMR to monitor lipid oxidation products. Unfortunately, chlorohydrin generation cannot be easily monitored but is only reflected by a broadened resonance of the original PL with limited information [218]. By contrast, PC and LPC can be easily differentiated by ^{31}P NMR, and even the position of the fatty acyl residue in the LPC species can be resolved.

8.4.2. Spectroscopic Methods. Although infrared (IR) spectroscopy is surely suitable for the detection of N-Cl vibration bands which would be expected if PE or PS reacted with HOCl, there have so far been no attempts to study chloramines by IR. However, it could be shown that the reaction between for example, taurine, and HOCl is accompanied by the formation of an intense IR band at about 975 cm^{-1} [219], which is characteristic of N-Cl. This band is characteristic enough to confirm the presence of chlorinated compounds.

The standard method for following the reaction between HOCl and amines is UV spectroscopy, because mono- and dichloramines are characterized by a (weak) absorption in the UV range at about 254 nm [220]. Nevertheless, there is a problem if amphiphilic compounds such as lipids are investigated: although UV spectroscopy can be easily used in the case of water soluble compounds, for instance, phosphorylethanolamine or glycero-phosphorylethanolamine [65], the reliability of this assay is limited when PEs are investigated due to the light scattering effects caused by the formation of aggregates in an aqueous environment. This requires the application of suitable detergents to minimize unwanted aggregation.

8.4.3. Chromatographic Methods. As already outlined (vide supra) HPLC is widely used to separate lipid oxidation products. Although TLC seems a bit old-fashioned in comparison to HPLC, it is also widely used in lipid research, as it offers many advantages, is inexpensive, and can also be performed by nonexperts [221]. Basically, the same separations performed by HPLC may also be accomplished by TLC. For instance, the effect of HOCl on cholesterol could be monitored by TLC [194] using normal phase TLC and diethyl ether, petroleum ether, and acetic acid (70/30/1, v/v/v) as eluent, while four different reaction products (the α- and β-chlorohydrins as well as the corresponding epoxides) could be monitored in addition to cholesterol. This also applies for more complex mixtures. Oxidation products of membranes from red blood cells could be detected by TLC although the authors were not satisfied with the TLC sensitivity and therefore also made use of an ELISA approach, which is more sensitive and could be successfully applied as soon as 1×10^6 cells were available [153]. Nowadays, this sensitivity problem could also be overcome by combining TLC with MALDI MS detection, which provides a much higher sensitivity in comparison to TLC alone [222]. Applications of TLC have recently been reviewed in the context of plasmalogen oxidation product analysis [211].

8.4.4. Immunological Methods. Such methods are widely applied to monitor the HOCl-induced modification of proteins by measuring, for instance, halogenated tyrosine residues [223] and, consequently, to determine the related MPO activities [224]. However, there are also antibodies available which recognize chlorinated fatty acids or lipids: in one of the first study an antibody against the chlorohydrin of oleic acid was developed [225], although this antibody was not very specific and also recognized some other chlorinated lipids. This method was also used to investigate the modification of red cell membrane lipids by HOCl [153], but otherwise this method is not frequently used.

9. Conclusions

Nowadays, it is commonly accepted that inflammation is accompanied by the generation of ROS and RNS. Since the majority of these reactive species are transient products and/or yield transient products as primary products, the establishment of reliable and specific "biomarkers" is still a challenging task.

The focus of this review was on the reaction between HOCl that is (i) used as an important disinfectant and (ii) generated under inflammatory conditions under catalysis of the enzyme MPO and different lipids. Nevertheless, the reactions of HOCl with some other biomolecules were also discussed, because they have functional groups which possess a higher reactivity than lipids. It was shown that lipids with exclusively olefinic residues (i.e., without reactive headgroups) yield mainly the corresponding chlorohydrins. Due to the stability of these reaction products, they may be easily characterized by various methods (particularly soft ionization mass spectrometry), and even the second order rate constants (k) of the reactions could be determined. Comparable results are obtained when HOCl is generated by the $MPO/H_2O_2/Cl^-$ system mimicking physiologically relevant conditions. This reaction is important for two reasons: (i) Chlorohydrin formation may lead to a destabilization of the cellular membrane and (ii) lipid peroxidation may be initiated by the reagent HOCl. Additionally, it was shown that higher unsaturated lipids also yield LPC, even in the complete absence of phospholipases. This might be an explanation of the enhanced LPC contents in joint fluids in patients with rheumatoid arthritis, which are also characterized by elevated MPO activities. A variety of chlorinated lipids can be produced by the reagent HOCl or the complete $MPO/H_2O_2/Cl^-$ system, but only α-chloro fatty aldehydes have so far been firmly demonstrated to occur during cardiovascular diseases, while evidence for other chlorinated lipids in disease and inflammation is still lacking. Since α-chloro fatty aldehydes can exclusively be generated by plasmalogen oxidation, this emphasizes the important role of these alkenyl ether lipids and the interest plasmalogens is currently attracting.

Compared to the reactions of cholesterol and PCs, the reaction between HOCl and lipids with reactive amino residues (e.g., PE and PS) has been by far less frequently investigated, although these lipids are also very abundant in biological membranes. This might be caused by the considerable product variability under these conditions, as the chloramines which are generated as the primary products are transient products and decay into aldehydes and nitriles but may also modify other functional residues of biomolecules.

Therefore, further attempts are necessary to study these reactions in more detail. This particularly applies as it was shown very recently that chlorinated products derived from PE and PS may impact the signal transduction pathways in cells.

List of Abbreviations

AACL: Amino acid chloramine
AP: Atherosclerotic plaque
ATP: Adenosine triphosphate
ATX: Autotaxin
α-ClFALD: α-Chloro fatty aldehyde
COPD: Chronic obstructive pulmonary disease
CP: Culprit plaque
EI: Electron ionization (electron impact)
EIA: Enzyme Immunoassay
ELISA: Enzyme-Linked Immunosorbent Assay
ESI: Electrospray ionization
EPR: Electron paramagnetic resonance
FFA: Free fatty acid
FID: Flame ionization detector
GAG: Glycosaminoglycan
GC: Gas chromatography
HOBr: Hypobromous acid
HOCl: Hypochlorous acid
HOI: Hypoiodous acid
HOSCN: Hypothiocyanite
HNE: 4-Hydroxy-2-nonenal
HPLC: High Performance Liquid Chromatography
HUVEC-ST: Immortalized human umbilical vein endothelial cells
IHC: Immunohistochemistry
IR: Infrared
k: Second order rate constant
LC: Liquid chromatography
LDL: Low-density lipoprotein
LPA: Lysophosphatidic acid
LPC: Lysophosphatidylcholine
LPL: Lysophospholipid
MALDI: Matrix-assisted laser desorption and ionization
MPO: Myeloperoxidase
MS: Mass spectrometry
m/z: Mass over charge
NADPH: Nicotinamide adenine dinucleotide phosphate
NET: Neutrophil extracellular trap
NICI: Negative ion chemical ionization
NMR: Nuclear magnetic resonance
NaOCl: Sodium hypochlorite
Nrf2: Nuclear factor erythroid 2-related factor 2
oxPAPC: Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine
PA: Phosphatidic acid
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PFB: Pentafluorobenzyl
PL: Phospholipid
PLA_1: Phospholipase A_1
PLA_2: Phospholipase A_2
PMNs: Polymorphonuclear leukocytes
PPARs: Peroxisome proliferator-activated receptors
PS: Phosphatidylserine
PUFA: Polyunsaturated fatty acid
RCS: Reactive chlorine species
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SF: Synovial fluid
SNP: Single Nucleotide Polymorphism
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid reactive substances
(HP)TLC: (High performance) thin-layer chromatography
TMB: 3,3′,5,5′-Tetramethylbenzidine
TNB: 5-Thio-2-nitrobenzoic acid
TOF: Time-of-flight
UV: Ultraviolet
VSMC: Vascular smooth muscle cell.

Disclosure

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Competing Interests

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

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Research Article

Behavior of Oxidative Stress Markers in Alcoholic Liver Cirrhosis Patients

Marina Galicia-Moreno,[1,2] Dorothy Rosique-Oramas,[1] Zaira Medina-Avila,[1] Tania Álvarez-Torres,[1] Dalia Falcón,[1] Fátima Higuera-de la tijera,[3] Yadira L. Béjar,[4] Paula Cordero-Pérez,[5] Linda Muñoz-Espinosa,[5] José Luis Pérez-Hernández,[3] David Kershenobich,[1,6] and Gabriela Gutierrez-Reyes[1]

[1]*HIPAM Lab, Experimental Medicine Unit, School of Medicine, Universidad Nacional Autónoma de México, Hospital General de México, Mexico City, Mexico*
[2]*Departamento de Farmacología, Facultad de Medicina Mexicali, UABC, Mexicali, BC, Mexico*
[3]*Liver Clinic, Gastroenterology Service, Hospital General de México, Mexico City, Mexico*
[4]*Blood Bank Service, Hospital General de México, Mexico City, Mexico*
[5]*Liver Unit and Molecular Medicine, University Hospital, Universidad Autónoma de Nuevo León, Monterrey, NL, Mexico*
[6]*Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico*

Correspondence should be addressed to Gabriela Gutierrez-Reyes; gabgurey@yahoo.com.mx

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Alcohol is the most socially accepted addictive substance worldwide, and its metabolism is related with oxidative stress generation. The aim of this work was to evaluate the role of oxidative stress in alcoholic liver cirrhosis (ALC). This study included 187 patients divided into two groups: ALC, classified according to Child-Pugh score, and a control group. We determined the levels of reduced and oxidized glutathione (GSH and GSSG) and the GSH/GSSG ratio by an enzymatic method in blood. Also, protein carbonyl and malondialdehyde (MDA) content were estimated in serum. MDA levels increased in proportion to the severity of damage, whereas the GSH and GSSG levels decreased and increased, respectively, at different stages of cirrhosis. There were no differences in the GSH/GSSG ratio and carbonylated protein content between groups. We also evaluated whether the active consumption of or abstinence from alcoholic beverages affected the behavior of these oxidative markers and only found differences in the MDA, GSH, and GSSG determination and the GSH/GSSG ratio. Our results suggest that alcoholic cirrhotic subjects have an increase in oxidative stress in the early stages of disease severity and that abstinence from alcohol consumption favors the major antioxidant endogen: GSH in patients with advanced disease severity.

1. Introduction

Alcohol is the most socially accepted addictive substance worldwide. The consumption of alcoholic beverages is a hallmark of social gatherings. However, in many societies, the consumption of these beverages in excess represents serious health and economic problems [1]. Chronic or excessive alcohol consumption can put physical and mental health at risk, damaging different organs such as the brain, liver, heart, lungs, skeletal musculature, and bones [2–4].

About 2–10% of absorbed alcohol is eliminated via the lungs and kidneys; the remainder is metabolized primarily by oxidative pathways in the liver and by nonoxidative pathways in the extrahepatic tissues. Oxidative metabolism in the liver is the result of extensive displacement of the liver's normal metabolic substrates, the production of acetaldehyde and reactive oxygen species (ROS), and an increase in the NADH/NAD^+ ratio [5]. Data that demonstrate an increase in ROS production and a decrease in the antioxidant enzyme glutathione peroxidase-1 strongly

suggest that chronic ethanol consumption creates an oxidative and potentially injurious environment within the hepatocyte, which could ultimately lead to oxidation and inactivation of cellular macromolecules. Lipid peroxidation [5] and oxidative alterations of mitochondrial DNA [6] have been observed after acute and chronic ethanol exposure. The pathogenic importance of the peroxidative process in ethanol-induced liver damage is still a subject of controversy. The positive evidence of enhanced lipid peroxidation in the liver has only been shown when animals are chronically fed with ethanol and given acute high doses of ethanol after overnight fasting or superimposed with a hypothermic condition [7]. In fact, only a few studies have examined the parameters of lipid peroxidation and hepatic content of antioxidants under a chronically intoxicated state.

Proteins are also an important target for oxidative damage because ROS can oxidize amino acid residues, cleave peptide bonds, increase protein fragmentation and aggregation, and alter proteolysis rates [5]. Thus, protein oxidation has to be considered one of several ethanol-related modifications that alter the functionality of proteins within the hepatocyte and especially within the mitochondrion, because ethanol increases ROS within this organelle. Protein modification elicited by the direct oxidative attack on the amino acid side chains by lipid peroxidation products, or as a consequence of reducing sugar, can lead to the generation of carbonyl groups within proteins [8]. On the other hand, reduced glutathione (GSH) is currently one of the most studied antioxidants. GSH is a natural compound made in the body from the amino acids glutamic acid, cysteine, and glycine. This molecule plays a crucial role in the body's detoxification process that occurs inside cells, mainly cells of the liver, kidney, intestines, and lungs. GSH has an especially important relationship with lipid peroxidation because of the known ability of such compounds to combine with free radicals that may initiate lipid peroxidation, as well as reduced hydrogen peroxide formed in cells [9]. Hepatic GSH has been observed to decrease after chronic alcohol consumption; this can be caused by acetaldehyde accumulation. Moreover, reduced GSH synthesis could also be a contributing factor to GSH depletion, as it has been documented in cirrhotic livers [10, 11]. The relationship between hepatic GSH and ethanol lipid peroxidation is unclear. GSH depression has been associated with ethanol-induced lipid peroxidation [11], but the depression may be either a result or a cause of the peroxidation. Table 1 summarizes the oxidative markers produced by alcohol consumption quantified in this work.

The aim of the present work was to analyze whether oxidative stress had an important role in alcoholic liver disease. This was done by quantifying liver damage through the use of different oxidative markers. We found that oxidative molecules played an important role during the course of alcoholic liver disease; this role was more evident in the lipid damage and glutathione markers in patients with liver cirrhosis.

Table 1: Biochemical markers of liver damage.

Liver damage markers	Level of damage
(i) MDA levels 4-Hydroxy-2,3-nonenal 4-Hydroxy-2,3-alkenal	(i) Damage in cellular membranes or lipid level
(ii) Carbonylated protein levels	(ii) Damage in biologically active proteins
(iii) GSH and GSSG quantification	(iii) Cytoplasmic damage

MDA: malondialdehyde; GSH: reduced glutathione; GSSG: oxidized glutathione.

2. Materials and Methods

2.1. Patients. Alcohol dependence and abuse were assessed with the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria and hazardous consumption was evaluated by the Alcohol Use Disorders Identification Test (AUDIT).

This cross-sectional study included 187 patients, eighteen years of age or older, who were divided into two groups: the control group ($n = 130$), which consisted of subjects with ethanol consumption ≤ 10 g/day and an AUDIT score ≤ 7, and the alcoholic liver cirrhosis (ALC) group ($n = 57$), which was made up of patients that presented with alcoholism in accordance with the World Health Organization (WHO) (ethanol consumption ≥ 70 g/day in men and ethanol consumption ≥ 50 g/day in women in the last 5 years) and a diagnosis of cirrhosis of the liver. The ALC group was then divided into 3 subgroups according to the patient Child-Pugh score: Child-Pugh A ($n = 22$), Child-Pugh B ($n = 26$), and Child-Pugh C ($n = 9$) [12].

Evaluation procedures included a detailed physical examination with anthropometry and assessment of the stigmata of nutritional deficiency.

Exclusion criteria for all groups were a positive viral panel, other concomitant liver damage, mental retardation, history of traumatic brain injury with loss of consciousness exceeding 10 min, and the presence of diseases that could affect the central nervous system.

The procedure was approved by the institutional review board. All participants provided written informed consent, and the study was carried out according to the provisions of the Declaration of Helsinki.

2.2. Chemicals. Trichloroacetic acid (TCA), hydrochloric acid, ethyl acetate, thiobarbituric acid, guanidine hydrochloride, and bovine albumin were purchased from Sigma Chemical Company (St. Louis, MO, USA). 2,4-Dinitrophenylhydrazine (DNPH), ethylic ethanol, and potassium dihydrogen phosphate were obtained from J.T. Baker (Xalostoc, Mexico).

2.3. Sample Collection. Blood samples (5 mL) were collected by venipuncture into Vacutainer tubes, with clot activators for serum collection and EDTA for blood and plasma collection

for the biochemical assays and oxidative stress evaluation. Blood was immediately centrifuged for 10 minutes at 1308 ×g to collect the serum and the plasma.

Total blood was also utilized for glutathione determination.

2.4. Biochemical Analysis. Biochemical testing included blood analysis and liver function tests and was performed with automated systems (Vitros 250, Johnson & Johnson, New Jersey, USA, and Beckman Coulter HMX-AL Hematology Analyzer, California, USA). Results were negative for hepatitis B surface antigen (HBsAg) and hepatitis C virus (HCV) antibodies.

2.5. Reduced Glutathione, Oxidized Glutathione, and GSH/GSSG Ratio Determination in Whole Blood. We measured blood levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) and the GSH/GSSG ratio by an enzymatic method using a commercially available kit (catalog number 371757, Calbiochem, Darmstadt, Germany) following the manufacturer's instructions. In short, the samples used to determine GSSG (100 μL of whole blood mixed with 10 μL of 1-methyl-2-vinylpiridinium trifluoromethane [scavenger]) and GSH (50 μL of whole blood) were immediately frozen until their determination. Both samples were thawed and then mixed and incubated at room temperature for 2–10 minutes. The samples were acidified with 5% metaphosphoric acid and the supernatant was separated by centrifugation at 1000 ×g for 10 min at 4°C. For GSH and GSSG determination, we employed Ellman's reagent, which reacts with GSH to form a product detectable by spectrophotometry at 412 nm. GSSG could be determined by reducing GSSG to GSH, which was then determined by their action with Ellman's reagent. This method utilizes the change in color that occurs during the reaction, and the reaction rate is proportional to the GSH and GSSG concentrations. The calculation of the GSH and GSSG concentrations and the GSH/GSSG ratio requires four steps: (a) determination of the reaction rate, (b) calibration curves, (c) analyte concentration, and (d) calculation of the GSH/GSSG ratio. The GSH/GSSG ratio was calculated according to the following formula: (GSH-2GSSG)/GSSG [13]. The GSH/GSSG ratio decreases as a consequence of GSSG accumulation. Measurement of the GSSG level or determination of the GSH/GSSG ratio is a useful indicator of oxidative stress.

2.6. Carbonylated Protein Determination in Serum. DNPH was used for determining the carbonyl content in proteins. 200 μL of 1 : 100 serum dilution was mixed with 200 μL 20% TCA; the samples were mixed and then centrifuged (3290 ×g for 3 minutes). After centrifugation, the supernatant was decanted and 0.5 mL of 10 mM DNPH was added to the protein pellet. Sample blanks were prepared using 0.5 mL of 2.5 N HCl. The tubes were placed in a dark environment for one hour at room temperature and vortexed every 15 minutes; 0.5 mL of 20% TCA was added to each tube and then centrifuged (3 minutes at 3290 ×g). After centrifugation, the supernatant was decanted and 1 mL of ethanol-ethyl acetate solution was added. Following the mechanical disruption of the pellet by vortexing, the tubes were allowed to stand for 10 minutes and then spun again (3 minutes at 3290 ×g). The supernatant was decanted and the pellet washed with ethanol-ethyl acetate two more times. After the final wash, the protein was solubilized in 1 mL of 6 M guanidine hydrochloride and 20 mM potassium dihydrogen phosphate (pH 2.3). To speed up the solubilization process, the samples were incubated in a 37°C water bath for 15 minutes. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 360, 370, and 390 nm and an absorption coefficient = 22000 M^{-1} cm^{-1} [14].

2.7. Lipid Peroxidation Assessment. Lipid peroxidation was estimated in the serum samples by measuring the malondialdehyde (MDA) formation using the thiobarbituric acid method [15]. Briefly, 100 μL of serum of alcoholic patients or control subjects was mixed with 500 μL of 150 mM Tris-HCl and 1.5 mL of 0.375% TBA and vortexed for 10 seconds. The reaction mixture was then incubated at 100°C for 45 minutes in a water bath. At the end of incubation, the samples were centrifuged at 1000 ×g for 10 minutes. The MDA content was calculated from the absorbance measurement at 532 nm and an absorption coefficient = 1.56×10^5 cm^{-1} M^{-1}.

Total protein was determined according to Bradford [16], using bovine serum albumin as the standard.

2.8. Statistical Analysis. Data were expressed as mean values ± SEM. Comparisons were carried out by analysis of variance (ANOVA) and orthogonal contrasts were used to determine the differences between all groups. Correlations were calculated with Spearman's rank correlation, as required. The analyses were carried out with the Windows SPSS 15.0 statistical software (SPSS Inc., Chicago, IL). Differences were considered statistically significant when the p value was less than 0.05.

3. Results

Table 2 shows the main demographic characteristics of our study population. We included 130 participants in the control group and 57 patients with alcoholic liver cirrhosis that were classified into subgroups, according to the Child-Pugh score, as Child-Pugh A, Child-Pugh B, or Child-Pugh C. Men predominated in all the study groups. The grams of ethanol consumed in a day for the patients with ALC were higher than those of the control group ($p \leq 0.05$). There was also a significant difference in relation to age between the Child-Pugh subgroups of the ALC patients; older patients had more severe disease ($p < 0.05$). Body mass index values are also shown in Table 2.

Serum markers of liver damage (AST, GGT, and albumin) were significantly altered according to liver damage progression (Table 3). The AST and GGT enzyme levels were higher in the ALC patients, compared with the control group, and the results were statistically significant ($p \leq 0.05$). Differences

Table 2: Results of clinical parameters of the subjects included in the study.

	Control	Patients with ALC			
		ALC	Child-Pugh A	Child-Pugh B	Child-Pugh C
Sex, F/M (%)	39/91 (30/70)	2/55 (3.5/96.4)	1/21 (1.8/36.8)	1/25 (1.8/43.8)	0/9 (0/15.7)
Age (years)	38.0 ± 1.4	49.3 ± 1.2*	45.9 ± 2.7**	47.2 ± 1.9***	53.2 ± 3.5******
Body mass index (kg/m^2)	28.3 ± 0.3	28.8 ± 0.6	29.5 ± 1.1	27.9 ± 0.8	29.9 ± 1.9
Consumption (g OH/day)	0.9 ± 0.1	304.1 ± 29.5*	372.3 ± 66.6**	257.9 ± 25.9***	271.1 ± 41.3****

Values represented as the mean ± SEM. $^{*}p \leq 0.05$, control group versus ALC group; $^{**}p \leq 0.05$, control group versus Child-Pugh A subgroup; $^{***}p \leq 0.05$, control group versus Child-Pugh B patients; $^{****}p \leq 0.05$, control group versus Child-Pugh C subgroup; $^{******}p \leq 0.05$, Child-Pugh A subgroup versus Child-Pugh C patients.

Table 3: Results of biochemical parameters evaluated in serum and blood of the study participants.

	Control	Patients with ALC			
		ALC	Child-Pugh A	Child-Pugh B	Child-Pugh C
AST (UI/L)	29.9 ± 0.9	50.2 ± 3.3*	37.7 ± 2.5**	50.5 ± 3.5***,*****	78.5 ± 14.01****,******
ALT (UI/L)	27.8 ± 1.6	33.1 ± 2.3	31.4 ± 3.2	34.4 ± 4.03	33.7 ± 4.1
GGT (UI/L)	31.5 ± 2.4	110.7 ± 13.1*	79.3 ± 13.4**	126.9 ± 20.8***	133.8 ± 44.1****
Albumin (g/dL)	4.4 ± 0.03	3.2 ± 0.1*	3.9 ± 0.08**	2.9 ± 0.1***,*****	2.3 ± 0.2****,******,*******
Hb (g/dL)	16.4 ± 0.1	12.8 ± 0.4*	13.8 ± 0.6**	12.4 ± 0.5***	11.8 ± 1.1****
Hct (%)	49.6 ± 0.3	38.5 ± 1.1*	41.9 ± 1.6**	36.9 ± 1.6***,*****	35 ± 3.01****
Platelets (mm^3)	270.5 ± 4.9	139.5 ± 12.3*	144.3 ± 20.08**	147.2 ± 20.7***	105.8 ± 11.4****

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; Hb: hemoglobin; Hct: hematocrit. Values represented as the mean ± SEM. $^{*}p \leq 0.05$, control group versus ALC group; $^{**}p \leq 0.05$, control group versus Child-Pugh A subgroup; $^{***}p \leq 0.05$, control group versus Child-Pugh B patients; $^{****}p \leq 0.05$, control group versus Child-Pugh C subgroup; $^{*****}p \leq 0.05$, Child-Pugh A versus Child-Pugh B subgroups; $^{******}p \leq 0.05$, Child-Pugh A subgroup versus Child-Pugh C patients; $^{*******}p \leq 0.05$, Child-Pugh B patients versus Child-Pugh C subgroup.

were also observed between the Child-Pugh A versus Child-Pugh B subgroups and the Child-Pugh A versus Child-Pugh C subgroups in regard to AST levels ($p \leq 0.05$). Table 3 also shows the levels of hemoglobin, hematocrit, and platelets. A directly proportional decrease in the levels of each marker was observed in all the cases with respect to the severity of damage and these results were statistically significant.

Various studies *in vitro* and *in vivo* suggest that oxidative stress plays an important role in the development of ALC [17]. Table 4 shows the results of several oxidative damage markers. The differences between MDA, GSH, and GSSG levels of the control group and the patients with cirrhosis of the liver were statistically significant. The MDA levels increased in proportion to the severity of damage, whereas the GSH and GSSG levels decreased and increased, respectively, in the Child-Pugh A subgroup but recovered at other stages of cirrhosis (Child-Pugh B and Child-Pugh C). There were no differences in the GSH/GSSG ratio and carbonylated protein content between groups. In addition, to evaluate whether active consumption of or abstinence from alcoholic beverages by the patients affected the behavior of these oxidative markers, we classified the patients according to the following criteria: 11 patients (Child-Pugh A), 9 patients (Child-Pugh B), and 6 patients (Child-Pugh C) were active consumers in the last year, and 11 patients (Child-Pugh A), 17 patients (Child-Pugh B), and 3 patients (Child-Pugh C) were abstainers (patients with no alcohol consumption during the last 6 months). Similar to results shown in Table 4, there were no significant differences between study groups in relation to carbonylated protein content (Figures 1(a) and 1(b)). On the other hand, as shown in Figure 2, the MDA levels increased in the nondrinking ALC patients and also in accordance with the Child-Pugh score ($p \leq 0.05$). With respect to the control group (Figure 2(a)), MDA concentration was higher according to liver disease severity and the pattern was the same in the ALC patients with active alcohol consumption (Figure 2(b)).

GSH behavior in all cirrhotic patients was different, regardless of active alcohol consumption or abstinence. In the Child-Pugh A subgroup, GSH levels decreased significantly in the abstainers, as well as in the active consumers, compared with the control group. The levels of this tripeptide recovered in the Child-Pugh B patients with and without alcohol consumption, but they decreased once more in patients with active alcohol consumption (Child-Pugh C subgroup) (Figures 3(a) and 3(b)). Conversely, Figures 4(a)

Table 4: Results of oxidative stress markers determined in serum and total blood of the study participants.

	Control	Patients with ALC			
		ALC	Child-Pugh A	Child-Pugh B	Child-Pugh C
Sex, F/M (%)	39/91 (30/70)	2/55 (3.5/96.4)	1/21 (1.8/36.8)	1/25 (1.8/43.8)	0/9 (0/15.7)
Carbonylated proteins (nmol carbonylated prot./mg prot.)	0.05 ± 0.007	0.05 ± 0.007	0.05 ± 0.01	0.04 ± 0.01	0.08 ± 0.01
MDA (nmol MDA/mg prot.)	0.1 ± 0.01	0.2 ± 0.02*	0.18 ± 0.02**	0.2 ± 0.02***	0.3 ± 0.1****
GSH (μM)	530.6 ± 9.1	475.5 ± 40.3	191. 3 ± 56.3**	683.1 ± 31.3***,*****	570.3 ± 37.7******,*******
GSSG (μM)	207.8 ± 16.6	368.9 ± 40.4*	663.3 ± 29.01**	176.8 ± 44.1*****	204.6 ± 80.8******
GSH/GSSG ratio	2.1 ± 0.2	0.1 ± 1.5	−0.5 ± 2.2	−0.5 ± 2.8	3.8 ± 1.2

Values represented as the mean ± SEM. $^{*}p \leq 0.05$, control group versus ALC group; $^{**}p \leq 0.05$, control group versus Child-Pugh A subgroup; $^{***}p \leq 0.05$, control group versus Child-Pugh B patients; $^{****}p \leq 0.05$, control group versus Child-Pugh C subgroup; $^{*****}p \leq 0.05$, Child-Pugh A versus Child-Pugh B subgroups; $^{******}p \leq 0.05$, Child-Pugh A subgroup versus Child-Pugh C patients; $^{*******}p \leq 0.05$, Child-Pugh B patients versus Child-Pugh C subgroup.

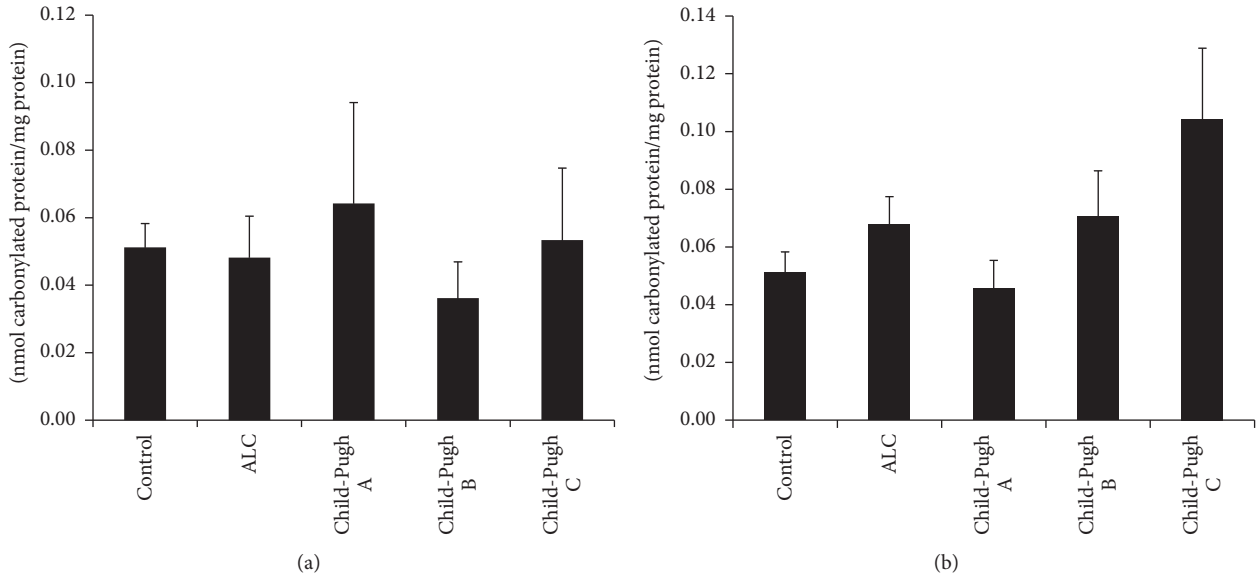

Figure 1: Carbonylated protein determination. Oxidative damage determined as carbonylated protein levels in serum from participants of the control group (control) and patients with alcoholic liver cirrhosis (ALC) classified according to the Child-Pugh score as Child-Pugh A, Child-Pugh B, or Child-Pugh C. (a) Patients without current alcohol consumption; (b) patients with active alcohol consumption. Each bar represents the mean value ± SEM. Differences were considered statistically significant when the p value was less than 0.05.

and 4(b) show that GSSG increased during the development of liver damage, being the highest in patients classified with Child-Pugh A, with respect to the control group, as well as to the Child-Pugh B and Child-Pugh C patients. This response was similar for both abstinence from alcohol and its active consumption (Figures 4(a) and 4(b)). Finally, the GSH/GSSG ratio had a positive direction in the abstinent patients ($p \leq 0.05$), showing that they had a higher concentration of reduced glutathione (Figure 5(a)). This response was the opposite in the active consumers (Figure 5(b)), indicating that there was a tendency to have a higher concentration of oxidized glutathione. Therefore, active alcohol consumption had a tendency to produce glutathione oxidation, whereas abstinence from alcohol consumption restored the main antioxidant molecule, reduced glutathione.

We found that the Child-Pugh Score was directly related to AST ($r_s = 0.522$, $p < 0.001$), albumin ($r_s = 0.707$, $p < 0.001$), MDA ($r_s = 0.395$, $p = 0.002$), GSH ($r_s = 0.589$, $p < 0.001$), and GSSG ($r_s = -0.657$, $p < 0.001$). The GSH levels were related to albumin ($r_s = -0.484$, $p < 0.001$) and the GSSG ($r_s = -0.546$, $p < 0.001$) and MDA levels with VCM ($r_s = -0.458$, $p < 0.001$) and GSSG ($r_s = 0.277$, $p = 0.037$).

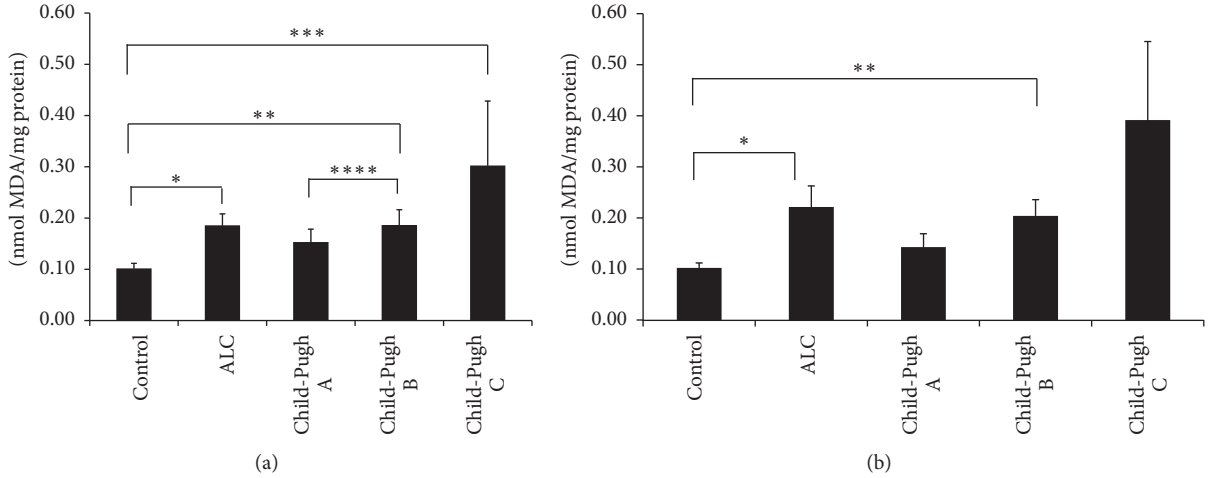

Figure 2: Evaluation of oxidative damage in lipids. Lipid peroxidation determined as malondialdehyde (MDA) content in serum samples from participants of the control group (control) and patients with alcoholic liver cirrhosis (ALC), classified according to the Child-Pugh score as Child-Pugh A, Child-Pugh B, or Child-Pugh C. (a) Patients without current alcohol consumption; (b) patients with active alcohol consumption. Each bar represents the mean value ± SEM. For (a): $^{*}p = 0.001$, control group versus ALC group; $^{**}p = 0.004$, control group versus Child-Pugh B subgroup; $^{***}p = 0.003$, control group versus Child-Pugh C subgroup; $^{****}p = 0.049$, Child-Pugh A versus Child-Pugh B subgroup. For (b): $^{*}p = 0.036$, control group versus ALC group; $^{**}p = 0.013$, control group versus Child-Pugh B subgroup.

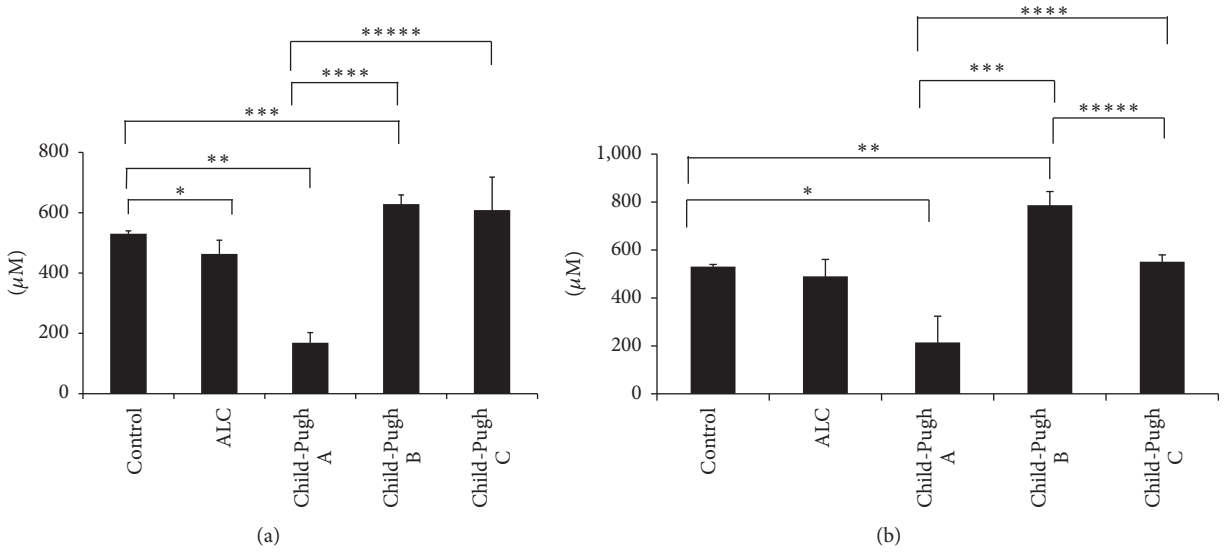

Figure 3: GSH level determination. Reduced glutathione (GSH) content was determined in total blood from participants of the control group (control) and patients with alcoholic liver cirrhosis (ALC), classified according to the Child-Pugh score as Child-Pugh A, Child-Pugh B, or Child-Pugh C. (a) Patients without current alcohol consumption; (b) patients with active alcohol consumption. Each bar represents the mean value ± SEM. For (a): $^{*}p = 0.022$, control group versus ALC group; $^{**}p = 0.001$, control group versus Child-Pugh A subgroup; $^{***}p = 0.001$, control group versus Child-Pugh B patients; $^{****}p = 0.001$, Child-Pugh A versus Child-Pugh B subgroups; $^{*****}p = 0.001$, Child-Pugh A subgroup versus Child-Pugh C patients. For (b): $^{*}p = 0.016$, control group versus Child-Pugh A subgroup; $^{**}p = 0.02$, control group versus Child-Pugh B patients; $^{***}p = 0.001$, Child-Pugh A versus Child-Pugh B subgroups; $^{****}p = 0.012$, Child-Pugh A subgroup versus Child-Pugh C patients; $^{*****}p = 0.003$, Child-Pugh B patients versus Child-Pugh C subgroup.

4. Discussion

ALD is a common response to and consequence of long-term ethanol abuse and represents a major cause of morbidity and mortality worldwide. The pathophysiology of this damage involves different stages, including steatosis, steatohepatitis, fibrosis, and cirrhosis, and a small percentage of patients with established cirrhosis develop hepatocellular carcinoma [18]. The mechanisms involved in ALC pathogenesis are immune and inflammatory responses, genetic factors, and the oxidative stress generated during hepatic alcohol metabolism.

Different studies have reported on oxidative stress participation in ALC. In 1963, Di Luzio [19], followed by other studies, showed that ethanol promotes the formation of a

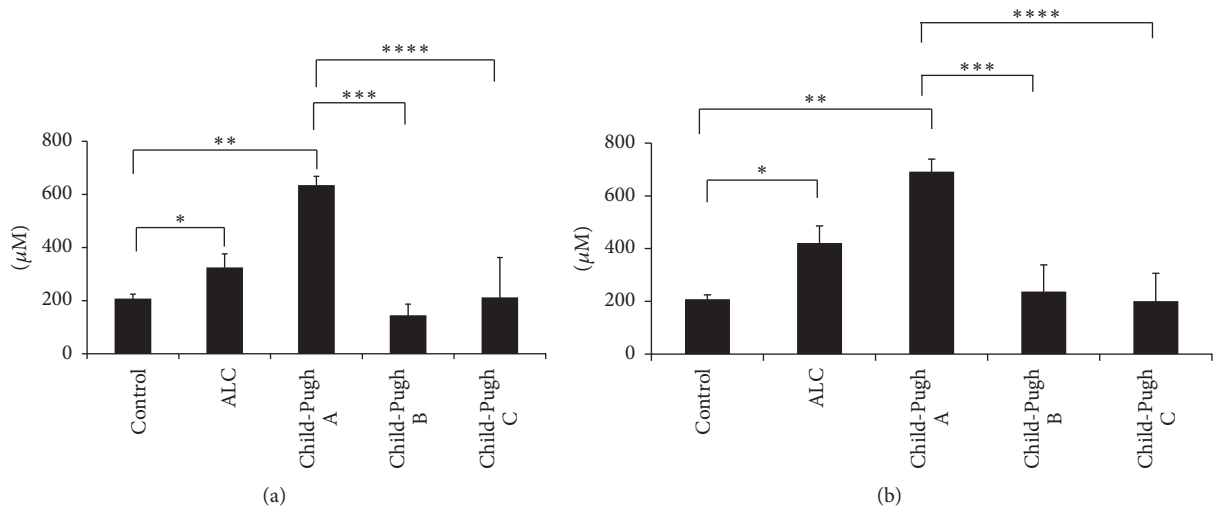

Figure 4: GSSG level determination. Oxidized glutathione (GSSG) levels were determined in total blood from participants of the control group (control) and patients with alcoholic liver cirrhosis (ALC), classified according to the Child-Pugh score as Child-Pugh A, Child-Pugh B, or Child-Pugh C. (a) Patients without current alcohol consumption; (b) patients with active alcohol consumption. Each bar represents the mean value ± SEM. For (a): $^{*}p = 0.008$, control group versus ALC group; $^{**}p = 0.001$, control group versus Child-Pugh A subgroup; $^{***}p = 0.001$, Child-Pugh A versus Child-Pugh B subgroups; $^{****}p = 0.001$, Child-Pugh A subgroup versus Child-Pugh C patients. For (b): $^{*}p = 0.001$, control group versus ALC group; $^{**}p = 0.001$, control group versus Child-Pugh A subgroup; $^{***}p = 0.001$, Child-Pugh A versus Child-Pugh B subgroups; $^{****}p = 0.001$, Child-Pugh A subgroup versus Child-Pugh C patients.

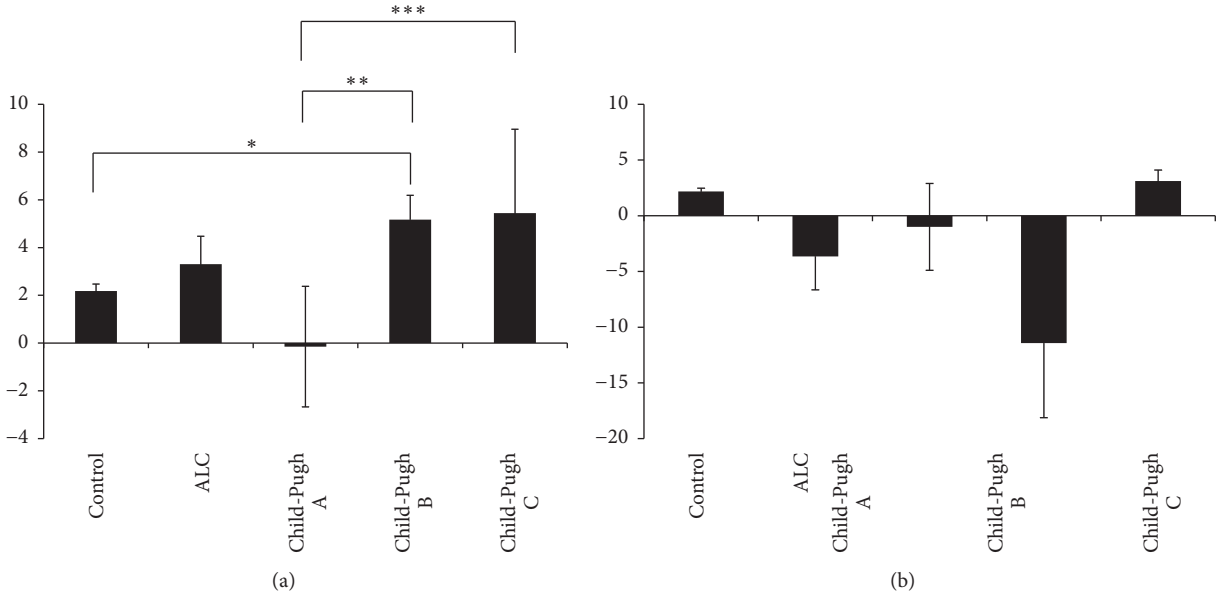

Figure 5: GSH/GSSG ratio determination. The GSH/GSSG ratio was determined in total blood from participants of the control group (control) and patients with alcoholic liver cirrhosis (ALC), classified according to the Child-Pugh score as Child-Pugh A, Child-Pugh B, or Child-Pugh C. (a) Patients without current alcohol consumption; (b) patients with active alcohol consumption. Each bar represents the mean value of experiments performed ± SEM. For (a): $^{*}p = 0.004$, control group versus Child-Pugh B patients; $^{**}p = 0.001$, Child-Pugh A versus Child-Pugh B subgroups; $^{***}p = 0.034$, Child-Pugh A subgroup versus Child-Pugh C patients.

variety of free radical intermediates by several cell types, including hepatocytes, Kupffer cells, endothelial cells, and infiltrating inflammatory leucocytes [20, 21]. Several studies have also demonstrated that supplementation with different antioxidants and free radical scavengers reduced hepatic injury in alcohol-fed rodents [22–24]. In the present work, we studied the behavior of several oxidative stress markers in serum and blood samples of patients with ALC, in specific liver cirrhosis that was also classified according to the Child-Pugh score.

Different reports indicate that oxidative stress, specifically oxidative mitochondrial damage, can be responsible for

hepatocyte apoptosis/necrosis in ALC [25]. This explains the results obtained in the ALT, AST, and GGT levels. The oxidative stress induction within liver mitochondria is associated with the collapse of the mitochondrial membrane potential and the onset of mitochondrial permeability transition (MPT) [26]. MPT is characterized by the opening of a megachannel in the mitochondrial membrane as a result of protein complex assembly [27]. Extensive MPT leads to mitochondrial swelling due to the influx of ions and water and is critical for the onset of hepatocyte death by necrosis [28]. Such a response explains the increase in liver damage markers we found in our patients. GGT is an enzyme derived from the plasma membrane of hepatocytes and its activity has been accepted as a biomarker of ALC [29]. This enzyme is involved in the transfer of γ-glutamyl peptides to amino acids and in the synthesis of GSH, hydrolyzing GSH to its amino acid components. The enzyme cysteine is used for intracellular resynthesis [30, 31] and thus plays an important role in the antioxidant defense system.

Our results showed that GGT activity increased and GSH levels decreased in the ALC patients. The GSH reduction in the Child-Pugh A patients was more evident than in the Child-Pugh B or Child-Pugh C patients. This decrease in the GSH levels could be explained by the years of alcohol consumption, according to Child-Pugh A, Child-Pugh B, and Child-Pugh C, which were 28, 26, and 22 years, respectively (data not shown).

Alcohol consumption may contribute to secondary anemia due to its direct effects on the liver and also to other different mechanisms [32]. Folic acid and vitamin B12 deficiencies frequently develop in patients with cirrhosis. These changes may be related to inadequate food intake or intestinal malabsorption. Folic acid deficiency is the most common cause of low hematocrit in alcoholic patients [33, 34]. Anemia in an alcoholic person may also be a consequence of the direct toxic effects on the erythrocyte precursor in bone marrow [35]. These observations may explain our study results in which the levels of hemoglobin and hematocrit decreased in relation to the severity of damage.

Ethanol consumption leads to the generation of ROS, which can potentially damage any biological molecules (proteins, lipids, or DNA). However, proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts, rather than stoichiometric mediators. Hence, the effect of damage to one molecule is greater than a stoichiometric change [36]. Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation [17, 37]. Carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized. These moieties are chemically stable, which is useful for both their detection and storage [36]. In our study, carbonylated protein levels did not show statistically significant differences between the control group and the patients with ALC (Child-Pugh A, Child-Pugh B, or Child-Pugh C), even taking into account the current consumption or abstinence of our participants.

We also evaluated lipid peroxidation through malondialdehyde quantification. Polyunsaturated lipids are essential to the entire support system of the cell, including cell membranes, the endoplasmic reticulum, and the mitochondria. Disruption of their structural properties can therefore have dire consequences on cellular function. Peroxidation of lipids has been thought to be a major effect of free radicals. Because of this, many of the assay methods for establishing free radical-induced injury have measured products of the reaction of these molecules with lipids; one of these products is malondialdehyde [38, 39]. The pathogenic importance of this peroxidative process in ethanol-induced liver injury is subject to debate. In fact, only a few studies have examined the parameters of lipid peroxidation and hepatic content of antioxidants under a chronically intoxicated state. Drinking more than two standard drinks per day over a long period of time may be associated with significant elevation of iron overload in tissue [40]. In animal models, iron and alcohol have been shown to act in a synergistic manner to enhance lipid peroxidation, leading to the formation of MDA [10]. Furthermore, it is known that patients with ALC have antibodies targeting cytochrome P450 2E1 and oxidized phospholipids. Preclinical and clinical studies show that the elevation of IgG against lipid peroxidation-derived antigens is associated with TNF-α elevation and the severity of liver inflammation [41]. Our results demonstrated that the serum levels of MDA were significantly higher in patients with cirrhosis of the liver and in the same order in subjects with different Child-Pugh scores. In regard to both active alcohol consumption and abstinence from alcohol, peroxidative damage was maintained.

There is much information about the effect of ethanol consumption and antioxidant defense depletion. GSH is the most important nonenzymatic antioxidant present in cells. Early studies have shown that a decrease in GSH levels and an increase in GSSG levels in the liver, regardless of nutritional status or the extent of liver disease, are a common feature in ethanol-fed animals, as well as in patients with alcoholism [19]. GSH homeostasis is very important in the prevention of alcohol-mediated oxidative injury. This statement is supported by the observation that the stimulation of GSH resynthesis in rats by supplementation with GSH precursors, such as N-acetylcysteine, prevents liver damage in the enteral alcohol model [30]. In our study, we observed the progressive changes in the levels of GSH, GSSG, and the GSH/GSSG ratio. In the first phases of liver damage, all parameters were altered, the most evident of which was the oxidative stress (the concentration of oxidized glutathione was higher than reduced glutathione as an outcome of GSSG accumulation) in patients with Child-Pugh A. It is known that short periods of alcohol consumption stimulate GSH depletion [11, 12], but Tietze in 1969 observed that, in long-term alcohol feeding in rats, the GSH levels increased. The mechanism of this response is unclear, but evidence suggests that depletion or increased turnover of glutathione due to ethanol is caused by the formation of acetaldehyde adducts [21], impaired glutathione synthesis [42], or increased losses from the liver tissue [43]. Our results of patients with ALC according to the Child-Pugh score were similar to those obtained in preclinical studies. Perhaps this was a compensatory response to counteract the alcohol damage

in long-term alcohol consumption (drinkers for at least 22 years).

Null alcohol consumption modified the behavior of GSH and GSSG, in particular in Child-Pugh B and Child-Pugh C patients. They showed increased GSH levels and lower GSSG concentration when compared with the Child-Pugh A patients. In addition, the GSH/GSSG ratio values were positive in the Child-Pugh B and Child-Pugh C groups, as a consequence of GSH accumulation, which favors antioxidation (Figures 3–5) and possibly stops different inflammatory mediators, such as cytokines. However, the GHS/GSSG ratio in patients with active consumption showed a negative trend, which is manifested as the presence of oxidative liver damage. These results must be confirmed in a larger number of patients in each Child-Pugh group. The number of patients in the present study was a limitation.

The free radical-antioxidant imbalance is possibly one of the major factors that contributes to the prevalence of mortality in the Mexican population, even though alcohol consumption *per capita* is moderate, compared with that of European countries [44].

Oxidative stress markers have normally been measured in liver tissue. Our results indicate that it is possible to measure them in peripheral blood, reflecting what is occurring in the liver in patients with alcohol liver cirrhosis.

Given our findings, we consider it necessary to carry out clinical trials that evaluate the use of antioxidants and the use of antioxidant therapy as a possible support therapy plus treatment to counteract liver damage induced by alcohol consumption.

5. Conclusions

Our results are the first to be reported on oxidative markers in a Latin American population. They suggest that alcoholic cirrhotic subjects have an increase in oxidative stress in the early stages of disease severity and that abstinence from alcohol consumption favors the major antioxidant endogen: GSH in patients with advanced disease severity.

Disclosure

Marina Galicia-Moreno was a fellow of the DGAPA-UNAM Postdoctoral Fellowship Program.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Marina Galicia-Moreno contributed to planning, interpreting the results, determining GSH, GSSG, GSH/GSSG ratio, and MDA levels, and writing of the manuscript. Zaira Medina-Avila was responsible for patient recruitment and sample collection. Dalia Falcón contributed to patient recruitment, sample collection, and MDA determination. Dorothy Rosique-Oramas contributed to the GSH and carbonylated protein determination. Fátima Higuera-de la tijera, Yadira L. Béjar, Paula Cordero-Pérez, Linda Muñoz-Espinosa, and José Luis Pérez-Hernández contributed to the review of the clinical records of all participants. David Kershenobich contributed to conducting the study. Gabriela Gutierrez-Reyes was responsible for planning and conducting the study. All the authors have approved the final manuscript.

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Review Article

Dysfunction of Autophagy: A Possible Mechanism Involved in the Pathogenesis of Vitiligo by Breaking the Redox Balance of Melanocytes

Zhuhui Qiao, Xiuxiu Wang, Leihong Xiang, and Chengfeng Zhang

Department of Dermatology, Huashan Hospital, Fudan University, Shanghai, China

Correspondence should be addressed to Chengfeng Zhang; e3dangdang@hotmail.com

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Vitiligo is a common chronic acquired pigmentation disorder characterized by loss of functional melanocytes from the epidermis and follicular reservoir. Among multiple hypotheses which have been proposed in the pathogenesis of vitiligo, autoimmunity and oxidative stress-mediated toxicity in melanocytes remain most widely accepted. Macroautophagy is a lysosome-dependent degradation pathway which widely exists in eukaryotic cells. Autophagy participates in the oxidative stress response in many cells, which plays a protective role in preventing damage caused by oxidative stress. Recent studies have enrolled autophagy as an important regulator in limiting damage caused by UV light and lipid oxidation, keeping oxidative stress in a steady state in epidermal keratinocytes and maintaining normal proliferation and aging of melanocytes. Impairment of autophagy might disrupt the antioxidant defense system which renders melanocytes to oxidative insults. These findings provide supportive evidence to explore new ideas of the pathogenesis of vitiligo and other pigmentation disorders.

1. Introduction

Vitiligo is an acquired pigmentary disorder of the skin and mucous membranes that is characterized by circumscribed, depigmented macules and patches. The worldwide prevalence of vitiligo ranges between 0.5 and 2% [1]. The highest incidence of the condition has been recorded in India, followed by Mexico and Japan [2]. Although vitiligo does not affect individual survival of patients, the change of the appearance may cause physical handicaps which seriously interfere with the lives of patients [3, 4]. In the past decades, a number of mechanisms such as oxidative stress, autoimmune, autocytotoxicity [5, 6], melanocytorrhagy [7], neural, and genetic factors [7] have been proposed for the pathogenesis of vitiligo. Oxidative stress hypothesis indicated imbalanced redox state of the vitiliginous skin. This results in the dramatic production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). ROS oxidize components of cells, leading to melanocyte destruction and creating depigmented macules [8]. Autoimmune theory showed that melanocytes are killed by autoimmune effector mechanisms, either memory cytotoxic T cells or autoantibodies directed to melanocyte surface antigens, as a result of tolerance breakage [9]. It is widely known that vitiligo can be associated with several autoimmune diseases, including autoimmune thyroid diseases [10], alopecia areata [11], halo nevi [12], and Addison's disease [13]. Furthermore, it has been shown that autoimmune thyroid diseases, especially Hashimoto thyroiditis, are the most common vitiligo-associated disorders [14]. The melanocythorragic hypothesis was proposed by Gauthier et al. in 2003 and they mentioned that nonsegmental vitiligo (NSV) occurs due to "melanocytorrhagy" or a chronic melanocyte detachment and loss caused by trauma and other stressors including catecholamines, ROS, or autoimmune elements [15]. This theory combined the concepts from other theories mentioned before to elaborate a single integrated explanation of vitiligo pathogenesis. The neural hypothesis suggested that accumulation of a neurochemical substance decreases melanin production. Abundant norepinephrine (NE) as an extrinsic factor secreted by nerve endings or keratinocytes leads to direct impairment of melanocytes differentiated from neural crest cells [16]. The link between vitiligo

and the activity of monoaminergic systems has been found based on the finding that levels of norepinephrine (NE), epinephrine (E), normetanephrine (NMN), metanephrine (MN), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5-HIAA) were significantly higher in patients with vitiligo compared to controls [17]. The current dogma is that there is a genetic component that renders the melanocyte fragile and susceptible to apoptosis that in turn predisposes individuals to developing the disease. Precipitating factors including sunburn, pregnancy, stress, and exposure to cytotoxic compounds can induce the fragile melanocytes to initiate programmed cell death or apoptosis when compared with the normal melanocyte [18]. Among the theories mentioned above, oxidative stress plays an extremely important role in the onset and progression of the disease [19]. In this article, we aim to summarize the latest findings concerning the association among oxidative stress, autophagy, and vitiligo.

2. Oxidative Stress and Vitiligo

Upon excessive oxidative stress, human bodies may suffer from various harmful factors, including highly active molecules such as active oxygen free radicals and ROS, which leads to impairment of redox balance and results in tissue damage. ROS can attack melanocytes and interfere with normal metabolism, proliferation, and differentiation of melanocytes, causing cell apoptosis and defects [20]. Increased levels of ROS might impair the function of mitochondrial. Excessive accumulation of ROS caused by chemical or physical stimulation in epidermis of vitiligo patients leads to destabilization, synthesis, and circulatory disorders of lipids in melanocytes, resulting in damage of mitochondria electron transport chain, and more ROS production, further forming a vicious circle and destroying melanocytes [21]. Patients with vitiligo accumulate up to 10^{-3} mol/L concentrations of H_2O_2 in their epidermis, which in turn affects many metabolic pathways in this compartment, including the synthesis and recycling of the cofactor (6R)-l-erythro-5,6,7,8-tetrahydrobiopterin (6BH4) [22]. In addition, it has been found that mitochondria appeared swollen and lack a normal and organized ultrastructure of internal and external membrane systems in keratinocytes from perilesional skin of vitiligo patients and the number of Langerhans cells increased in varying differentiative phases, in lesional skin of vitiligo patients, indicating that mitochondrial damage is associated with the increase in ROS production and, hence, oxidative stress decreased total antioxidant capacity [23]. Besides, alteration in the mitochondrial distribution exists in melanocytes from vitiligo patients, suggesting that the impaired mitochondrial activity may account for the increased susceptibility to prooxidants [24]. In addition, oxidative stress can increase the production of toxic intermediates during melanin synthesis, which promotes the release of catecholamine (CA) and then triggers the damage of melanocytes [25].

Among the great variety of ROS, H_2O_2 has a pivotal role in the onset and progression of vitiligo [5]. Several studies have shown the level of catalase decreased in the skin of vitiligo patients and the concentration of H_2O_2 in the epidermis was much higher than normal. Suction blister roofs taken from the involved and uninvolved epidermis of patients with vitiligo showed a consistent reduction in levels of catalase compared to normal healthy controls of matched photo-skin types. A decrease in catalase activity is expected to increase the concentration of hydrogen peroxide in the epidermis of these patients [26]. Elevated H_2O_2 levels were demonstrated in vitiligo patients compared with healthy controls by utilizing Fourier-transform Raman spectroscopy, providing in vivo evidence for epidermal H_2O_2 accumulation within the entire skin of patients with vitiligo. These results are strongly supported by the in vitro observations using epidermal cell extracts, as well as melanocyte and keratinocyte cell cultures established from lesional and nonlesional epidermis of patients with vitiligo [27].

Keratinocytes transfer H_2O_2 to the neighboring melanocytes [28], thereby inactivating various antioxidant enzymes, including catalase (CAT), glutathione S-transferase (GST), acetylcholinesterase (AchE), and methionine sulfoxide reductase A (MsrA). The activity of these enzymes in lesional skin of patients with vitiligo was significantly reduced [29–31], so as to further aggravate the accumulation of ROS. Studies have indicated that the destruction of antioxidant systems in patients with vitiligo made the melanocytes more vulnerable to damage caused by oxidative stress [32]. In contrast, other researches on oxidative stress in vitiligo showed that there was a statistically significant increase in the levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and other antioxidant enzymes in patients with vitiligo compared with control group [33]. Furthermore, some evidence confirmed that low concentration of H_2O_2 could affect the regulation of human pigmentation by increasing melanin synthesis and melanosome transfer, while this activity was downregulated by high concentrations of H_2O_2, suggesting that oxidation levels differentially impact melanocytes [34].

Keap1 (Kelch-like ECH-associated protein 1)/Nrf2 (nuclear factor (erythroid-derived 2)-like 2)/ARE (antioxidant responsive element) signaling pathway is the antioxidant response that provides cellular antioxidants and detoxifying enzymes [35]. The key transcription factor Nrf2 regulates the expression of phase II detoxifying and antioxidant genes by binding to the ARE sequence [36]. Under basal conditions, the transcription factor Nrf2 is located in the cytoplasm of the cell bound to Keap1 [37]. When cells are under oxidative stress, the inducers disrupt the cytoplasmic complex between the actin-bound protein Keap1 and the transcription factor Nrf2, thereby releasing Nrf2 to migrate to the nucleus where it activates ARE of phase II genes and accelerates their transcriptions [38]. Recent studies have identified an important role for the Nrf2 pathway in maintaining skin homeostasis in epidermis [39]. Phase II genes encode heme oxygenase-1 (HO-1), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC), and glutamyl cystine ligase modulatory subunit (GCLM) [40]. Previous study showed that Nrf2-ARE pathway and its

downstream antioxidants are crucial for melanocytes to cope with H_2O_2-induced oxidative damage [41]. Investigation on the integrity of phase II detoxification pathway in the epidermis of vitiligo patients showed that the transcript levels of Nrf2 as well as the downstream detoxification genes NQO-1, GCLC, and GCLM are upregulated in the lesional epidermis compared with the matched nonlesional skin [42]. Meanwhile, several evidences demonstrated that the induction of HO-1 by activating the Nrf2-ARE pathway protected melanocytes against H_2O_2-induced toxicity and that upregulation of Nrf2 expression diminished the H_2O_2-induced apoptosis of human melanocytes [43]. Furthermore, genetic studies in Chinese Han population demonstrated that Nrf2 gene polymorphisms are associated with the susceptibility to vitiligo [44, 45]. Taken together, such evidence indicated that the Nrf2-ARE signaling pathway is altered in vitiligo melanocytes and alteration of this pathway might be involved in the pathogenesis of vitiligo. Therefore, impairment of the Nrf2-ARE signaling pathway in vitiligo melanocytes might lead to a dysfunction of redox balance, which provides a reasonable explanation as to why the melanocytes in vitiligo patients are hypersensitive to H_2O_2-induced oxidative stress [41].

3. Oxidative Stress and Autophagy

Autophagy, also referred to as "self-eating," is a lysosome-dependent degradation pathway that widely exists in eukaryotic cells. In severe conditions such as hunger, infection, or stress, it can regulate the degradation of long-life proteins and organelles in cells and then the degradation products are recycled [46]. In addition, autophagy also plays an important role in cell growth, cell immunity, tissue remodeling, and adaptability to the environment [47]. There are roughly three classes of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is thought to be the major type of autophagy and it has been studied most extensively compared to microautophagy and chaperone-mediated autophagy [46]. Autophagy can be upregulated in response to extra or intracellular stress and signals such as starvation, growth factor deprivation, endoplasmic reticulum (ER) stress, accumulation of unfolded proteins, and infection [48]. One of the best characterized substrates of selective autophagy is p62, which is also known as sequestosome 1/SQSTM1. P62 is a ubiquitously expressed cellular protein, which is conserved in animals but not in plants and fungi [46]. Impairment of autophagy is accompanied by accumulation of p62. This leads to the formation of large aggregates, which include p62 and ubiquitin [49]. Functions of p62 may determine whether cells survive by activating the TNF receptor-associated factor 6- (TRAF6-) NF-kB pathway or die by facilitating the aggregation of caspase-8 and downstream effector caspases [50]. On the other hand, p62 interacts with the Nrf2-binding site on Keap1, a component of Cullin3-type ubiquitin ligase for Nrf2. This interaction stabilizes Nrf2 and activates the transcription of Nrf2 target genes, including a battery of antioxidant proteins [51, 52]. It is thus possible that excess accumulation or aggregation of p62 leads to hyperactivation of these signaling pathways [46].

Autophagy has been proved to participate in the removal of toxic molecules produced after oxidative stress. Oxidatively modified proteins are usually considered degraded more or less exclusively by the proteasome system. Rather, evidence suggests that moderately or heavily oxidized proteins are endocytosed and enter the endosomal/lysosomal system, indicating that the proteins modified by oxidizing stressors are degraded by the proteasome and autophagosomal/lysosomal pathways [53]. More and more evidence has been raised suggesting the balance between autophagy and oxidative stress is essential to maintain the function of cells, as well. He et al. found that short exposure to oxidative stress could induce autophagy in myocardial cells, while long-term oxidative stress stimulated the inhibition of autophagy, which leaded to the death of cardiac muscle cells. Therefore, stability of autophagy plays an important role in protecting different types of cells from oxidative stress [54]. Recent study demonstrated that the pathological changes observed in autophagy-defective livers were due, at least in part, to persistent activation of Nrf2 by the excess accumulation of p62, resulting in the appearance of destructive phenotypes and liver injury, proposing that, in such pathological conditions, the high levels of p62 associated with the suppression of autophagy might result in activation of Nrf2 [51].

Recently, autophagy in skin cells has been studied. External and internal sources of oxidative stress include UVR/IR, pollution (environment), lifestyle (exercise and diet), alcohol, and smoking all of which may potentially have impacts on skin [55]. All these factors, leading to an increase in ROS generation and/or a reduction in the antioxidant capacity, contribute to oxidative stress, which exposes the skin cells to the formation and accumulation of irreversibly damaged proteins, lipids, nucleic acids, and carbohydrates [56]. Human skin is exposed to environmental insults such as UV light that cause oxidative damage to macromolecules. In response to oxidative stress, cells activate the Nrf2 antioxidant response that provides cellular antioxidants and detoxifying enzymes [35]. Meanwhile, epidermal keratinocytes activate autophagy in response to UVA and UV-oxidized phospholipid [57]. Genetic elimination of autophagy resulted in massive accumulation of protein aggregates in stressed cells, elevation of Nrf2 target gene expression, and strikingly a significant rise in various oxidized species of phospholipids, implying that during homeostasis autophagy prevents accumulation of oxidized phospholipids, as well as overexpression of Nrf2 target genes in keratinocytes [57].

In autophagy-deficient cells, an accumulation of the adapter protein p62 can be observed frequently [57]. It has been described that lipoxidative stress can lead to protein aggregates that are positive for p62, so called "p62 bodies" in various tissues [51]. P62 is however not only the adapter between cargo and the autophagic machinery but also a component of the Nrf2 pathway. It is not only a target of Nrf2, but its accumulation can compete with Nrf2 for binding to its cytosolic anchor, which then leads to increased Nrf2 nuclear translocation and activity (Figure 1). Excessive accumulation of p62 in autophagy-deficient mice and excessive activation of

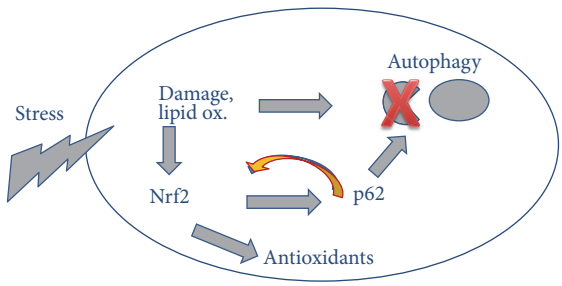

Figure 1: P62, a target gene AND activator of Nrf2.

Nrf2 pathway could cause liver cell damage, which is consistent with the disease such as drug hepatitis and hepatocellular carcinoma [51]. In addition, blockage of autophagy in mice keratinocytes or melanocytes led to substantial accumulation of high molecular weight protein p62, excessive activation of the antioxidant Nrf2 pathway, and high expression of a large number of harmful macromolecular protein and lipid oxidation of phosphoric acid [57, 58]. Thus, normal activation of the Nrf2 pathway can protect cells from oxidative insults, while long-term and excessive activation of the Nrf2 pathway can no longer protect the cells but cause tissue damage.

External and internal sources of oxidative stress expose the cells to the formation and accumulation of irreversibly damaged proteins and lipids, activating the Nrf2 antioxidant response that provides cellular antioxidants and detoxifying enzyme. Impairment of autophagy leads to accumulation of high molecular weight protein p62, which can compete with Nrf2 for binding to its cytosolic anchor, leading to increased Nrf2 nuclear translocation and activity. Excessive activation of the Nrf2 pathway may no longer protect the cells but cause tissue damage.

4. The Association among Autophagy, Oxidative Stress, and Vitiligo

Skin is the largest human organ and is directly exposed to environmental oxidant stress, including UVA and oxidized lipids generated by UV irradiation. Melanocytes settle down in the epidermis and act as the guard to photoprotection and thermoregulation by packaging melanin pigment into melanosome and delivering them to neighboring keratinocytes. Epidermal melanocytes are particularly vulnerable to excessive ROS production owing to their specialized function: melanin synthesis, which is stimulated by sun exposure, during the process of tanning, and by inflammation that results in postinflammatory hyperpigmentation [59]. Even though the mechanism of melanocyte loss in vitiligo has not yet been clarified, a rising number of studies provided evidence that dysfunction of autophagy may serve as a vital factor. Recently, more and more research has focused on the possible associations between autophagy and melanosome biogenesis, formation, and destruction [59, 60]. Small interfering RNA-based screens identified autophagy genes as having an impact on melanogenesis and heterozygosity for the autophagy regulator beclin-1 results in altered fur color of mice [61]. In addition, members of the autophagic machinery have been proposed to have a role in the formation and maturation of melanosome [62, 63]. However, direct evidence for this claim is still lacking. Murase et al. explored the involvement of autophagy in determining skin color by regulating melanosome degradation in keratinocytes. Melanosome accumulation in keratinocytes was accelerated by treatment with lysosomal inhibitors or with small interfering RNAs specific for autophagy-related proteins, which are essential for autophagy. Furthermore, consistent with the alterations in skin appearance, the melanin levels in human skin cultured ex vivo and in human skin substitutes in vitro were substantially diminished by activators of autophagy and enhanced by the inhibitors, revealing that autophagy has a pivotal role in skin color determination by regulating melanosome degradation in keratinocytes and thereby contributes to the ethnic diversity of skin color [60]. In addition, Zhang et al. showed that mouse melanocytes lacking the autophagy protein Atg7 undergo premature senescence in vitro and accumulate products of oxidative damage, despite activation of the redox response [58]. Taken together, evidence mentioned above indicated that autophagy has implications for melanocyte dysfunction and manifestations of skin pigmentary disorders.

In summary, oxidative stress is currently recognized as one of the most important theories concerning pathogenesis of vitiligo, while autophagy is involved in response to oxidative stress and plays a protective role in the damage induced by oxidative stress in melanocytes. Autophagy is required for suppressing the activation of the Nrf2-dependent stress response, maintaining the proliferative potential and preventing premature senescence of melanocytes. It has been described that controlled p62 levels and p62 phosphorylation are needed for ordered cell cycle progression, so hyperexpression of p62 may well interfere with cell cycle [64]. We speculate the accumulation of p62 and consequently Nrf2 dysregulation might be a sign of impaired redox household and cell cycle machinery and contribute to the observed decrease in the proliferative potential of autophagy-deficient melanocytes. Disturbance of autophagy might release the control of Nrf2 and indirectly triggers an antistress response. If this response is too strong, premature senescence is likely to be induced (Figure 2).

Under oxidative stress, ROS produced in melanocytes could induce autophagy and activate the Nrf2 antioxidant pathway, remove toxic molecules, and maintain the redox homeostasis of melanocytes, while continuous stimulation of ROS might eventually lead to inactivation of autophagy and excessive activation of Nrf2 antioxidant pathway and break the redox homeostasis of melanocytes, resulting in premature senescence, decreased proliferation, and pigment synthesis, which may be involved in the onset of vitiligo.

5. Conclusion

Oxidative stress plays an important role in initiating the destruction of melanocytes and impairment of redox balance could be one possible mechanism of vitiligo. Since autophagy possesses a protective and restorative function

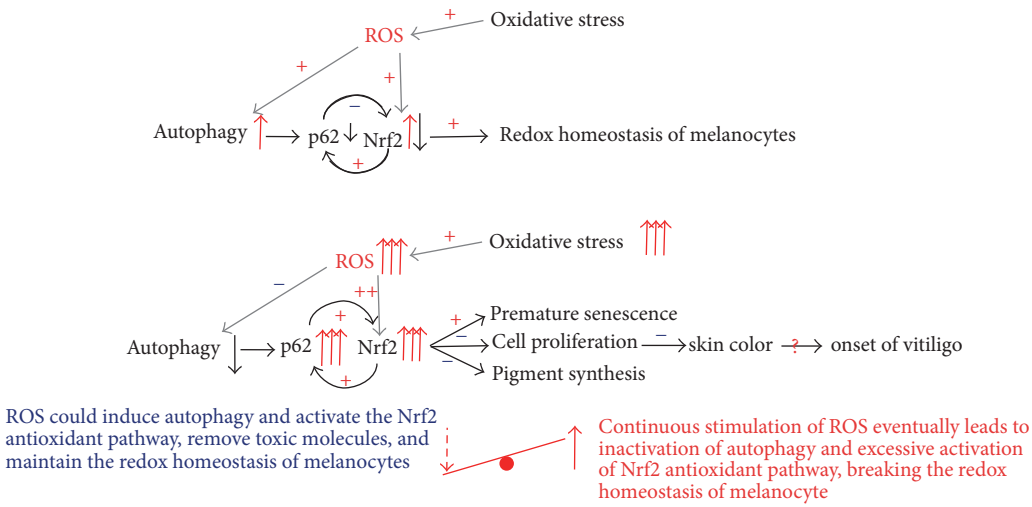

Figure 2: Oxidative stress, autophagy, and vitiligo.

to the damage induced by oxidative stress, deficiency of autophagy might lead to alteration of cell functions such as proliferation, senescence, and ROS scavenging. On the basis of the similarity between Atg7-deficient and vitiligo phenotypes, specifically with respect to the activation of Nrf2 regulated genes, oxidative stress, and premature senescence, it is very likely that autophagy-deficient melanocytes and vitiligo melanocytes share defective cellular redox regulation, increased membrane lipid oxidation, and premature senescence [65]. However, whether there exists dysfunction of autophagy in vitiligo patients and whether autophagy plays a role in the pathogenesis of vitiligo remain to be established. Additional studies are required to establish an unambiguous role for autophagy in melanocyte biology and skin pigmentation.

Competing Interests

The authors declare that they have no conflict of interests.

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Research Article

The Oxidative and Inflammatory State in Patients with Acute Renal Graft Dysfunction Treated with Tacrolimus

Sandra Carrillo-Ibarra,[1] José Ignacio Cerrillos-Gutiérrez,[2] Ariadna Escalante-Núñez,[2] Enrique Rojas-Campos,[3] Benjamín Gómez-Navarro,[2] Sonia Sifuentes-Franco,[1] Ernesto Germán Cardona-Muñoz,[1] and Alejandra Guillermina Miranda-Díaz[1]

[1]*Institute of Experimental and Clinical Therapeutics, Department of Physiology, University Health Sciences Centre, University of Guadalajara, Guadalajara, JAL, Mexico*
[2]*Department of Nephrology and Transplants, Specialties Hospital, National Occidental Medical Centre, The Mexican Social Security Institute, Guadalajara, JAL, Mexico*
[3]*Kidney Diseases Medical Research Unit, Specialties Hospital, National Occidental Medical Centre, The Mexican Social Security Institute, Guadalajara, JAL, Mexico*

Correspondence should be addressed to Alejandra Guillermina Miranda-Díaz; kindalex1@outlook.com

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Objective. To determine the oxidative stress/inflammation behavior in patients with/without acute graft dysfunction (AGD) with Tacrolimus. *Methods.* Cross-sectional study, in renal transplant (RT) recipients (1-yr follow-up). Patients with AGD and without AGD were included. Serum IL-6, TNF-α, 8-isoprostanes (8-IP), and Nitric Oxide (NO) were determined by ELISA; C-reactive protein (CRP) was determined by nephelometry; lipid peroxidation products (LPO) and superoxide dismutase (SOD) were determined by colorimetry. *Results.* The AGD presentation was at 5.09 ± 3.07 versus 8.27 ± 3.78 months ($p < 0.001$); CRP >3.19 mg/L was found in 21 versus 19 in the N-AGD group ($p = 0.83$); TNF-α 145.53 ± 18.87 pg/mL versus 125.54 ± 15.92 pg/mL in N-AGD ($p = 0.64$); IL-6 2110.69 ± 350.97 pg/mL versus 1933.42 ± 235.38 pg/mL in N-AGD ($p = 0.13$). The LPO were higher in AGD ($p = 0.014$): 4.10 ± 0.69 μM versus 2.41 ± 0.29 μM; also levels of 8-IP were higher in AGD 27.47 ± 9.28 pg/mL versus 8.64 ± 1.54 pg/mL ($p = 0.01$). Serum levels of NO in AGD were lower 138.44 ± 19.20 μmol/L versus 190.57 ± 22.04 μmol/L in N-AGD ($p = 0.042$); antioxidant enzyme SOD activity was significantly diminished in AGD with 9.75 ± 0.52 U/mL versus 11.69 ± 0.55 U/mL in N-AGD ($p = 0.012$). *Discussion.* Patients with RT present with a similar state of the proinflammatory cytokines whether or not they have AGD. The patients with AGD showed deregulation of the oxidative state with increased LPO and 8-IP and decreased NO and SOD.

1. Introduction

Chronic end-stage renal disease (ESRD) is characterized by serious, irreversible kidney damage distinguished by proteinuria and a reduction in the glomerular filtration rate (GFR) [1, 2].

According to the Registry of Dialysis and Transplants in the State of Jalisco (*Registro de Diálisis y Trasplante del Estado de Jalisco: REDTJAL*) and in the State of Morelos, the number of new patients who require renal replacement therapy (RRT) is constantly increasing [1, 2]. The inherent annual cost reported by the Ministry of Health in Mexico per patient with ESRD in 2012 was $8,966.00 USD while at the Mexican Social Security Institute (*Instituto Mexicano del Seguro Social: IMSS*) it was $9,091.00 USD [3]. The options available for the management of ESRD are peritoneal dialysis (PD), hemodialysis (HD), and renal transplantation (RT) [4]. Renal transplantation is considered the best therapeutic option for patients with ESRD [5]. The United States Renal Data System (USRDS) reports that the incidence rate of RT in Jalisco is among the highest in the world, based on the latest USRDS report where Taiwan, Jalisco (Mexico), and the

United States are indicated as the highest incidence of treated ESRD (458, 421, and 363 per million of population (pmp), resp.) [5].

The introduction of Cyclosporine A (CsA) followed by the rapid succession of Tacrolimus (TAC) together with Mycophenolate Mofetil (MMF) is associated with a much lower rate of loss of the renal allograft, achieving graft survival in >90% annually [6]. The most common and feared adverse effect of TAC is nephrotoxicity [7]. Among the greatest challenges in the management of RT is acute rejection (AR), although with new immunosuppressant strategies the frequency of AR has been reduced in the last decades, with significant long-term improvement in survival of the graft and the patient [8, 9]. In Mexico, survival of the graft and survival of the patient are both comparable to those reported by other countries [10, 11]. However, despite the efficacy of actual immunosuppressive regimes for the prevention of AR, nephrotoxicity and infections can influence the appearance of renal graft dysfunction [12]. Only in transplantation between identical twins could immunosuppressive therapy not be required: all RT recipients require immunosuppression in order to avoid rejection. Therefore, it is fundamental to find a balance between the immunosuppressor effect of the drugs and the immunological response of the host in order to prevent the appearance of opportunistic infections that cause the inflammatory state and acute dysfunction of the graft (AGD) [13]. The inflammatory state plays an important role in the interrelationship with oxidative stress (OS) in ESRD and in RT recipients [14, 15]. The OS is characterized by the imbalance between the generation of the oxidant and antioxidant systems. The primary sources of generation of reactive oxygen species (ROS) after RT are found in ischemia-reperfusion and immunosuppression. The reperfusion injury is a common phenomenon in the transplanted kidney and can cause dysfunction of the allograft during the first posttransplant week [16].

Levels of the proinflammatory cytokines like interleukin 6 (IL-6), the tumor necrosis factor alpha (TNF-α), the C-reactive protein (CRP), and the markers of OS have been reported as being significantly elevated in ESRD, with significant decreases two months after RT [17].

The AGD is defined as the slow and progressive deterioration of renal function habitually accompanied by proteinuria of varying degrees and hypertension. The most frequent causes of dysfunction are rejection mediated by antibodies, interstitial fibrosis, and tubular atrophy of unspecific origin. The diagnosis of the cause of AGD requires renal biopsy [18]. Since TAC can cause acute tubule-interstitial nephropathy and because the mechanisms of renal damage associated with immunosuppression are not entirely understood, we proposed the objective of determining the behavior of the markers of oxidative stress and inflammation in patients with AGD and without AGD (N-AGD), treated with TAC.

2. Patients and Methods

2.1. Study Design. An analytical cross-sectional study was performed. Two study groups were made of RT recipients within the first year of follow-up. The first group included all patients with AGD (elevation of creatinine ≥30%) biopsy proven (all biopsies were evaluated by the same pathologist and the diagnosis was recorded), in the period of Jan-2014 to Dec-2015; control group included patients without AGD (N-AGD); these patients were randomly obtained from the pool of patients who were unto protocolized graft biopsy (this is a common behavior in our setting); they were rejection-free at the time of biopsy. All patients were on triple immunosuppression scheme based on TAC, MMF, and Prednisone (considered as the most potent scheme). Both groups were first-time recipients of RT. The calculation of the sample size was determined using the formula to compare means and considering, as a variable in the determination, concentration of the superoxide dismutase enzyme (SOD), obtaining 55 patients per study group. The patients were attended to at the Department of Nephrology, Transplant Division, of the Sub-Specialties Medical Unit at the National Occidental Medical Centre of the Mexican Social Security Institute (*IMSS* in Spanish). The ingestion of TAC for immunosuppressant therapy was considered an inclusion criterion for both groups. Excluded were the patients >55 years of age, who presented with renal comorbidities, who received a second transplant, and who were undergoing treatment with nonsteroidal anti-inflammatories, angiotensin converting enzyme (ACE) inhibitors, and antagonists of the angiotensin II receptors (ARBs), as well as recipients of transplants from perished donors. The serum levels of IL-6, TNF-α, 8-isoprostanes (8-IP), and Nitric Oxide (NO) were determined with ELISA. Nephelometry was used to determine CRP and the colorimetric method was used to determine levels of the products of lipid peroxidation (LPO) (malondialdehyde (MDA) and 4-hydroxy-alkenals) and levels of the SOD enzyme as an antioxidant.

2.2. Biochemical Analysis. Once blood samples were collected in two separate tubes (one with 0.1% of ethylenediaminetetraacetic (EDTA) and the other a dry tube), the plasma and serum were separated by centrifugation at 2,000 rpm for 10 minutes at room temperature. Then, the samples were stored at −80°C until processing. All of the technical readings of optical density were made with the Synergy HT (BIOTEK®) microplate reader.

2.3. TNF-α and IL-6. TNF-α levels were determined by ELISA, following the instructions of the kit manufacturer (Peprotech, Rocky Hill, NJ 08553, USA). First, 100 μL of diluted capture antibody was added, followed by incubation overnight at room temperature. Then, 300 μL of block buffer was added to the wells and it was incubated for 1 hour at room temperature. Serum and standards were added, followed by incubation for 2 hours at room temperature. After several washings, 100 μL of diluted detection antibody was added and incubated at room temperature for 2 hours. 100 μL diluted Avidin-HRP conjugate was added, followed by incubation for 30 minutes at room temperature. Finally, 100 μL of substrate solution was added to each well. The plate was read at

a wavelength of 405 nm with correction set at 650 nm and was reported in pg/mL.

2.4. Human High Sensitivity C-Reactive Protein (hsCRP). Levels of the hsCRP in serum were assessed with immune nephelometry, using the BN II System (Siemens, USA). The reagents, controls SL/1 and SL/2, CardioPhase hsCRP (polystyrene particles coated with mouse monoclonal antibodies to CRP), and N rheumatology standard were prepared according to the manufacturer's instructions. The reference curve was made, and the samples were diluted 1 : 20 and mixed with the CardioPhase hsCRP. The hsCRP levels were measured automatically by the nephelometry BN II. The levels are reported automatically in mg/L.

2.5. Products of Lipid Peroxidation. Plasma LPO levels were measured using the FR22 assay kit (Oxford Biomedical Research Inc., Oxford, MI, USA) according to the manufacturer's instructions. In this assay the chromogenic reagent reacts with MDA and 4-hydroxy-alkenals to form a stable chromophore. First, 140 μL of serum with 455 μL of N-methyl-2-phenylindole in acetonitrile (Reagent 1) was diluted with ferric iron in methanol. Samples were agitated, after which 105 μL 37% HCl was added, followed by incubation at 45°C for 60 minutes and centrifugation at 12,791 rpm for 10 minutes. Next, 150 μL of the supernatant was added and absorbance was measured at 586 nm. The curve pattern with known concentrations of 1,1,3,3-tetramethoxypropane in Tris-HCl was used.

2.6. 8-Isoprostane (8-IP) Evaluation. The immunoassay reagent kit from Cayman Chemical Company® (Michigan, USA) was used according to the manufacturer's instructions. The 8-IP assay was based on the principle of competitive binding between sample 8-IP, 8-IP acetyl cholinesterase (AChE) conjugate, and 8-IP tracer. 50 μL of samples or standard was added to each well and 50 μL of 8-IP AChE tracer was added to all wells except the total activity and blank wells. 50 μL of 8-IP enzyme immunoassay antiserum was added to all wells except the total activity and blank wells. At once, 50 μL of 8-IP antiserum was added to all wells except total activity, nonspecific binding, and blank wells. The plate was covered and incubated at 4°C for 18 h and then washed 5 times with buffer. Absorbance was read at 420 nm.

2.7. Nitric Oxide (NO). The levels of NO in serum were assessed by sandwich ELISA using a commercially available kit (Human Total Nitric Oxide ELISA Kit, MyBioSource®, San Diego, CA, USA). Before performing the assay samples, reagents were kept at room temperature for 30 min. Nitric Oxide or serum (50 μL) was pipetted into an antibody-coated 96-well plate with 100 μL of HRP-conjugate reagent and incubated at 37°C for 1 h. The wells were then washed four times with buffer wash; 50 μL of chromogen solution A and 50 μL of chromogen solution B were added. The samples were incubated for 15 minutes at 37°C; then 50 μL of stop solution was added, and the absorbance was read at 450 nm.

2.8. Superoxide Dismutase. Serum total SOD activity (U/mL) was determined using a kit from Cayman Chemical Company®, (USA, number 706002), following the manufacturer's protocol, to detect the O^{2-} generated by the xanthine oxidase and hypoxanthine enzymes through the reaction of tetrazolium salts. The serum samples were diluted 1 : 5 in sample buffer, 200 μL of the radicals' detector (diluted 1 : 400) was placed, and 10 μL of the sample was added. After slow agitation, 20 μL of xanthine oxidase was added to the wells. The microplate was incubated for 20 minutes at room temperature, and the absorbency was read at a wavelength of 440 nm. Levels are reported in U/mL.

2.9. Ethical Considerations. The study was performed in accordance with the Principles of Ethics for Medical Research in Human Beings as stipulated by the Declaration of Helsinki 64th General Assembly, Fortaleza, Brazil (October 2013). Informed consent forms were signed, because it was a category III study, in agreement with the General Health Law in Mexico. The project was submitted to and approved by the local Scientific Research and Health Ethics Committee of the *IMSS* (Registration number R-2015-1301-83) and the State Research Registry (59/E-JAL/2015) put forth by the General Public Health Administration (*Dirección General de Salud Pública*) in Jalisco, Mexico.

2.10. Statistical Analysis. Continuous variables are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM) and were analyzed with nonparametric tests according to the results obtained by the Kolmogorov-Smirnov test. For the comparisons between groups the Mann–Whitney U test was used. The categorical variables are presented as frequencies and percentages and were analyzed with the Chi^2 test. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Demographic and Metabolic Characteristics. The AGD was developed at 5.09 ± 3.07 after transplant ($p < 0.001$) versus 8.27 ± 3.78 months in N-AGD (this was the time of follow-up for protocol biopsy). The age of patients with AGD was 25.39 ± 5.71 years and 28.08 ± 9.12 years in N-AGD. The male gender significantly predominated in both groups ($p = 0.004$): there were 48 (87%) males in the AGD group and 34 (62%) in the N-AGD group. Heights and weights between the AGD and N-AGD groups were not significantly different. Tobacco use (smoking) was present in 15 patients with AGD and in 8 N-AGD. Alcoholism was present in 14 patients with AGD and in 9 of the N-AGD. Differences in fasting glucose levels were not statistically significant. Findings of uremia were significantly increased in the AGD group with 54.78 ± 3.99 mg/dL ($p < 0.001$) versus the N-AGD with 36.59 ± 1.32 mg/dL, and the same behavior was found in levels of serum creatinine which was significantly higher in AGD with 1.19 ± 0.20 mg/mL ($p = 0.002$) versus 1.06 ± 0.27 mg/mL in N-AGD. The age of the donor was significantly higher in the AGD group with 42.54 ± 11.45 years ($p < 0.001$) versus

Table 1: Clinical characteristics, demographics, proinflammatory cytokines, oxidants, and antioxidants. In terms of the recipients, there were significantly more transplantations done in males than females. As a point of inclusion in the study the creatinine was found significantly elevated in AGD, as was urea. The significant older age of the donors could have influenced the AGD. It is attention-grabbing that the inflammatory state between AGD and N-AGD did not predominate. The oxidative state is characterized by significant increases in LPO and 8-IP in AGD and diminished NO and SOD activity.

	N-AGD	AGD	p
Demographic and metabolic characteristics			
Age (years)	28.08 ± 9.12	25.39 ± 5.71	0.116
Weight (kg)	62.21 ± 13.35	69.16 ± 22.37	0.241
Height (m)	1.69 ± 0.08	1.66 ± 0.10	0.398
Gender F/M, *n* (%)*	21/34 (38/62)	7/48 (13/87)	**0.004**
Glucose mg/dL	99.57 ± 3.37	100.73 ± 2.24	0.340
Urea mg/dL	36.59 ± 1.32	54.78 ± 3.99	<**0.001**
Cr habitual (mg/mL)	1.06 ± 0.27	1.19 ± 0.20	**0.002**
CT (mg/dL)	152.13 ± 29.16	145.41 ± 35.56	0.230
LDL (mg/dL)	77.96 ± 26.66	83.51 ± 25.46	0.214
HDL (mg/dL)	43.59 ± 10.82	43.22 ± 5.47	0.474
VLDL (mg/dL)	31.11 ± 21.37	35.11 ± 13.52	0.088
TAG (mg/dL)	150.41 ± 94.63	161.91 ± 72.78	0.128
Tobacco no/yes, *n* (%)*	47/8 (85/15)	40/15 (73/27)	0.147
Alcoholism no/yes, *n* (%)*	46/9 (83/17)	41/14 (76/24)	0.304
Donor age (years)	34.07 ± 10.65	42.54 ± 11.45	<**0.001**
Time after transplant (months)	8.27 ± 3.78	5.09 ± 3.07	<**0.001**
Proinflammatory cytokines			
TNF-α (pg/mL)	125.54 ± 15.92	145.53 ± 18.87	0.636
IL-6 (pg/mL)	1933.42 ± 235.38	2110.69 ± 350.97	0.129
CRP mg/L, *n* (%)*			
≤3.19	36 (65.5)	34 (61.5)	0.828
>3.19	19 (34.5)	21 (38.5)	
Oxidants			
LPO (μM)	2.41 ± 0.29	4.10 ± 0.69	**0.014**
8-isoprostanes (pg/mL)	8.64 ± 1.54	27.47 ± 9.28	**0.012**
Nitric Oxide (μmol/L)	190.57 ± 22.04	138.44 ± 19.20	**0.042**
Antioxidants			
SOD (U/mL)	11.69 ± 0.55	9.75 ± 0.52	**0.012**

AGD: acute graft dysfunction. Mean ± standard deviation or standard error, p = Mann–Whitney U test, and * p = Chi^2 test.

34.07 ± 10.65 years in N-AGD. The triglycerides and cholesterols were not significantly different between the groups (Table 1).

3.2. Proinflammatory Cytokines. Levels of high sensitivity CRP ≤3.19 mg/L were found in 34 AGD patients and >3.19 mg/L in 21 patients. In the N-AGD recipients, 36 patients had ≤3.19 mg/L and 19 had >3.19 mg/L, without significant differences ($p = 0.83$). The average serum level of TNF-α in AGD was 145.53 ± 18.87 pg/mL versus 125.54 ± 15.92 pg/mL in N-AGD, without a significant difference ($p = 0.64$). Serum levels of IL-6 in AGD patients were 2110.69 ± 350.97 pg/mL versus 1933.42 ± 235.38 pg/mL in N-AGD, which was not significantly different ($p = 0.13$) (Table 1).

3.3. Markers of Oxidative Stress. Serum levels of LPO in AGD were significantly elevated ($p = 0.014$, Mann–Whitney U test) with 4.10 ± 0.69 μM versus 2.41 ± 0.29 μM in N-AGD. A similar behavior was found in 8-IP since the average plasma level in AGD patients was also significantly elevated with 27.47 ± 9.28 pg/mL versus 8.64 ± 1.54 pg/mL in N-AGD ($p = 0.01$, Mann–Whitney U test).

However, it is noteworthy that the serum levels of NO in the AGD group were found to be significantly diminished with 138.44±19.20 μmol/L versus 190.57±22.04 μmol/L in N-AGD ($p = 0.042$, Mann–Whitney U test). The activity of the SOD enzyme was significantly diminished in the AGD group with 9.75 ± 0.52 U/mL versus 11.69 ± 0.55 U/mL in N-AGD ($p = 0.012$, Mann–Whitney U test) (Table 1).

3.4. Histopathology Results. From each AGD patient the histopathological diagnosis was recorded: 13% were normal; 45% had acute rejection; in 30% toxicity was found; and in

13% other diagnosis: acute tubular necrosis, and borderline changes [19].

4. Discussion

Renal transplantation is the RRT of choice for patients with ESRD, to provide them with better quality of life and the best survival compared to dialysis, as well as being the most cost-efficient [20]. Besides kidneys from live donors function much better than those from perished individuals. Donors to the AGD group were significantly older (42.54 ± 11.45 years); and although they were found in a state of perfect health, age could be an independent factor in the appearance of AGD. In this regard, it has been previously reported that the acceptable age of live kidney donors continues to be controversial due to the higher incidence of comorbidities and greater risk of postoperative complications [21, 22].

In our study as in our setting, male receptors are 3 : 1 compared to female [10, 23]; this is due to social and epidemiological conditions; perhaps our results show that AGD was most commonly presented in males; all AGD in the period of time were included; nevertheless gender was associated to it presence; due to the fact that all patients have the same immunosuppressive scheme it is possible that this finding was gender-related. Other studies have shown significant correlation in OS in males compared to females although the gender was not a condition to differentiate OS levels (AGD more frequent in men). On the other hand, there is clinical evidence that suggests that male's receptors from female donors could lose graft function [24], and it is well known that male gender is a risk factor for CKD; however, it is necessary to test the hypothesis of the possible association between gender and AGD [25]. In this study, the majority of patients who were subjected to RT were male: 87% of those with AGD and 62% of N-AGD.

One of the primary causes of later loss of the allograft is chronic graft nephropathy, which is characterized, in part, by deterioration of renal function. Registered data demonstrate that renal function in the first posttransplant year is an important predictor of the long-term result in how the RT behaves, where factors that initiate the cycle of the loss of nephrons play a predominant role. In this regard, advanced age, the masculine gender, or diabetes mellitus are considered, among other factors [21, 26].

It has been reported that serum creatinine concentrations of ≤ 1.5 mg/dL at 6–12 months are associated with greater survival of the graft at 5 years. These serum creatinine results can be predictive of survival as soon as 1 month after transplantation [27]. In patients included in this study AGD was detected between 5 and 8 months after transplant, which suggests that follow-up should be frequent during the first year. It is considered that the restoration of kidney function through RT improves the chronic inflammatory state of the OS associated with uremia, by contributing to improving the survival of patients and the transplanted organ.

The proinflammatory cytokine TNF-α is a functional transmembranal homotrimeric of 26-kDa that is liberated in circulation in functional soluble form of 17-kDa. In plasma TNF-α is free or it binds with its circulating receptor [28]. The TNF-α, in general, is not present in the kidneys. After stimulation by the lipopolysaccharides, the interleukin-1α, and during inflammation, the TNF-α and its receptors are expressed in the glomerular tuft (endothelial, mesangial, and epithelial) and in the tubular cells [29]. In the patients with AGD the levels of TNF-α were not significantly elevated ($p = 0.64$) compared to N-AGD.

The IL-6 in AGD behaved similarly with an increase that was not significant compared to the N-AGD group ($p = 0.13$). The IL-6 is a multifunctional pleiotropic pro-inflammatory cytokine of 26-kD molecular weight, with the ability to modulate local and systemic immunity [30]. The overproduction of IL-6 leads to the deposit of extracellular matrix proteins, the development of inflammatory lesions, and the synthesis of acute-phase proteins. However, we cannot assume the impact of the overproduction of these proinflammatory cytokines because in the present study their systemic expression was not significant between the groups [31]. With regard to the CRP results, we consider them ambiguous and that the value of the data in relation to the diagnostic precision of the CRP is limited [32]. Although IL-6, TNF-α, and CRP are wide studied in clinical studies regarding inflammation, all of them are predictive to mortality and other cardiovascular outcomes in RT patients; up to our knowledge this is the first evaluation of the possible association in AGD of inflammation and OS; however, it is necessary to evaluate other inflammatory markers that may play a role in AGD.

The oxidative state after RT is not entirely known. It is important to consider that oxygen is necessary for the life of aerobic organisms and that the univalent reduction of oxygen leads to the formation of ROS, like the O^{2-}. The mechanisms of cellular injury from free radicals involve proteins, lipids, enzymes, receptors, and, at the membrane level, the initiation of lipid peroxidation with the increase in LPO. Lipid peroxidation is put into play as a consequence of the formation of free radicals in the cells and tissues. It is one of the first aspects of abnormal oxidation. The study of the mechanisms of the adverse effects of the LPO identified as aldehydes of the 4-hydroxy-alkenal class [33, 34] is characterized by their high reactivity in the cellular components. In the present study, the LPO were significantly elevated in AGD, which could suggest that the patients with AGD are found in a process of abnormal oxidation.

In 1990, it was demonstrated that the production of a series of F2 compounds of the prostaglandin (known as F2 isoprostanes) that form *in vivo* and *in vitro* by free radicals catalyzes peroxidation of the phospholipid of the arachidonic acid, through an independent pathway of the cyclooxygenase. Although the isoprostanes are less reactive than the other products of peroxidation, they are liberated into circulation, which means that the determination of F2-isoprostanes in serum or plasma could be considered trustworthy markers of OS by lipid peroxidation, to evaluate the oxidative state in diverse human pathologies. The F2 isoprostanes and carbonyl associated protein are elevated in ESRD and descend after RT [17], but there is not any evidence in the AGD setting [14]. In

our study, the 8-IP was significantly elevated in AGD, which suggests that 8-IP could induce vasoconstriction effects in the kidney, giving way to alterations in renal function [35].

The endothelial NO synthase (eNOS) is identified as a very important protective factor in renal function [36].

In our study, the levels of NO in AGD were significantly diminished compared to the N-AGD group ($p = 0.042$), in detriment to the endothelial function of the RT, as well as the pathophysiological effects the NO has in relation to OS. The NO is produced from the L-arginine as a result of a catalytic reaction by the NOS enzyme. The NO can control such physiologic processes as important as blood pressure, arterial smooth muscle relaxation, platelet aggregation and adhesion, neurotransmission, and neuroendocrine secretion. It also participates in the destruction of pathogenic microorganisms and tumor cells from leukocytes and macrophages. These functions could be compromised by the diminishing of NO in AGD [37]. The relative scarcity of substance and of cofactors conducts the uncoupling of inducible NOS, resulting in the production of O^{2-} and the activation of transcription factors that later increase the expression of inducible NOS [38].

The diminished activity of the antioxidant enzyme SOD in AGD could mean the persistence of ROS, as was previously reported in a study that found decreased SOD during the early phase of RT that indicated a persistent source of ROS production and of OS. One possible reason could be the TAC-based treatment, since it has been reported that Cyclosporine (another calcineurin inhibitor like TAC) augments the glomerular synthesis of ROS [39].

It is important to consider that TAC remains as the major immunosuppressant drug, and its common adverse effects include arterial hypertension, hyperlipidemia, and hyperglycemia. Pharmacological action of TAC has been previously described; the only known effect of TAC in inflammation is proinflammatory, due to the elevation of ICAM-1 and VCAM-1 dose dependent [40]. This is the reason why TAC cause toxicity. There is no evidence regarding the possible effect of TAC in other inflammatory pathways, although this TAC has been known to decrease OS [41]. Our findings suggest that AGD is a state where OS is elevated and not necessarily due to TAC deficiency or inflammation. It was shown that calcineurin inhibitor exposure induces heat shock protein expression, decreased NO production in cultured tubular epithelial cells, and alterations in calcium influx and free cytosolic calcium concentration, further illustrating the direct toxic effects of calcineurin inhibition on tubular function [42]. Several studies indicate that vascular dysfunction by calcineurin inhibitor results from an increase in vasoconstrictor factors that include endothelin and thromboxane and activation of the renin-angiotensin system, as well as a reduction of vasodilator factors like prostacyclin, prostaglandin E2, and NO [43]. In addition, calcineurin inhibitor induces imbalances in the vasodilator/vasoconstrictor ratio of arachidonic acid metabolites (eicosanoids), which ultimately promotes renal vasoconstriction. The renal vasoconstriction can lead to renal hypoperfusion and hypoxia-reoxygenation injury and subsequently to the formation of ROS or free radicals, which causes cellular injury [44].

Cardiovascular disease is the most common mortality cause after RT; statins have shown a protective lipid lowering effect, but there is no evidence regarding the possible antioxidant effect of statins [45] in the RT setting. Our purpose was not to evaluate the effect of other drugs (instead TAC), that is, statins; it is necessary to design a study *ex profeso*, but there is no evidence regarding the possible pharmacological interaction among TAC and statins in OS [46, 47]. Also we have to consider that AGD is a multifactorial pathological state; OS is only one pathway in its development. To the best of our knowledge, there is no evidence regarding use of antioxidants agents to prevent/treat AGD in RT; due to the observational nature of our results we cannot assure if AGD is cause of consequence of OS; nevertheless a significant association is shown and can be considered as predictors and indicators of AGD. This study shows an opportunity to treat these patients. Our findings indicate that it is possible to consider the evaluation of the effectiveness of antioxidant treatment to prevent and/or treat AGD patients. Clinical studies should be performed to prove this hypothesis; perhaps the choice of antioxidant agent is one of the major challenges, due to pharmacological interactions, immunosuppression treatment, and other medication risks.

In conclusion, we found similar expression of proinflammatory cytokines in patients with and without AGD and deregulation of the OS in patients with AGD.

The limitations of the present study include a small sample size and it was a cross-sectional study. We are conscious that it is convenient to provide close follow-up in patients after modifying the dose of TAC in the first year and periodic long-term follow-up.

Competing Interests

The authors have no conflict of interests to report.

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