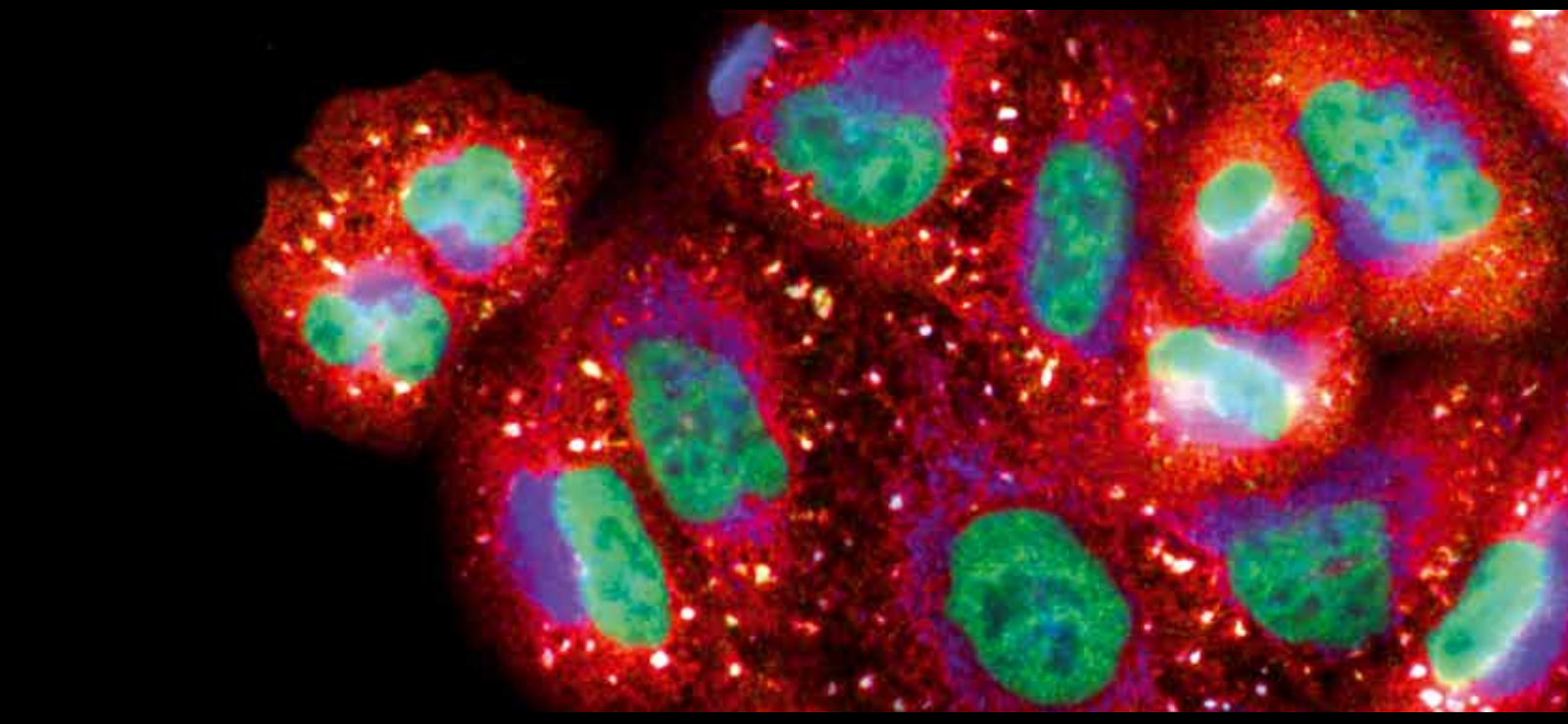
Oxidative Medicine and Cellular Longevity

Hypoxia-Induced Oxidative Stress in Health Disorders

Guest Editors: Vincent Pialoux, Damian Bailey, and Rémi Mounier



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Editorial Board

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Editorial

Hypoxia-Induced Oxidative Stress in Health Disorders

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Chronic hypoxia has been shown to promote a large number of pathologies such as hypertension, cardiovascular and metabolic disorders, and respiratory diseases. The oxidative stress that occurs during intermittent or continuous cellular hypoxia is likely involved in all these diseases. Indeed, the reactive species were demonstrated to inhibit active substances, modulate the signaling of intracellular pathways, and mediate enzymes activation, which are known to play a critical role in the geneses and/or the outcomes of these pathologies.

The four review articles of this special issue describe the current knowledge regarding the role of oxidative stress in ischemic retinopathy, pulmonary diseases, and infertile testis and discuss a new model of yeast to study hypoxia-induced oxidative stress. In addition, the two original research articles presented in this issue further expand the understanding of the redox biology in the context of pulmonary arterial hypertension and impaired spermatogenesis induced by hypoxia.

The review article by S.-Y. Li et al. presents the underlying mechanisms involved in the hypoxia/ischemia-induced oxidative damage in diabetic retinopathy and retinopathy of prematurity. In particular, the authors have discussed the effect of therapeutic strategies of antioxidants treatment such as administration of catalase and superoxide dismutase, vitamin E, and lutein and inhibition of NADPH oxidase or similar signaling pathways in these retinal ischemic diseases.

The extensive review by O. F. Araneda and M. Tuesta reports recent *in vitro* and *in vivo* experimental evidence that shows the implied mechanisms in pulmonary redox state by hypoxia via the increase of ROS generation in mitochondria, as from activation of NADPH oxidase, xanthine oxidase/reductase, and nitric oxide synthase enzymes, as well as throughout inflammatory process. Then, the role and impact of enzymatic and nonenzymatic antioxidant in the modulation of the pathways involved in the physiopathological response to hypoxia are extensively discussed. In a last part, the authors report the evolution of the two most studied makers of oxidative stress (exhaled nitric oxide for humans and pulmonary content of malondialdehydes for animals) in the lung in response to hypoxic exposure.

The paper of J. G. Reyes et al. synthesizes the current state of knowledge of the physiology of the testicles under pathologic hypoxic conditions that lead to reduced spermatogenesis. The molecular events triggered by all causes of hypoxia in the testis share common mechanistic pathways involving ROS generated by mitochondrial dysfunction and activation of enzymes such as xanthine oxidase or the inducible nitric oxide synthase. The recent results analyzed suggest that conditions such as germ cell apoptosis and DNA damage are common features in hypoxic testicles such as varicocele and testicular torsion. In addition, the oxidative damages present in hypoxia suggest the ROS play a role in the initiation stages of germ cell damage and apoptosis.

In another review by M. I. G. Siso and M. E. Cerdán the yeast *Kluyveromyces lactis* K. is proposed as a respiratory eukaryote model, complementary to the fermentative *S. cerevisiae*, for the study of the pathways of hypoxia-induced oxidative stress. Although these two yeasts share homology between their acting proteins, the experimental studies

described in this review reveal that there are many differences from a comparative perspective with respect to the signaling pathways and mechanisms of cells regulation including life span adjustment, programmed cell dead, autophagy, and mitophagy.

The paper by A. Zepeda et al. tests the protective and antioxidant effects of a blueberry extract in testis of rats exposed to hypobaric hypoxia. The authors demonstrated that the decreased apoptosis of the testicles cells observed with such supplementation was probably driven by a reduced lipid peroxidation and increased antioxidant enzymes activities. This suggests that blueberry extract may have a high antioxidant capacity and that its regular ingestion could prevent the testicles disorders related to oxidative stress induced by hypoxia.

Finally, M. Mata et al. address the relationship between the expression of the proliferator-activated receptor γ coactivator-1α (PGC-1α), the pulmonary vascular resistance, and the oxidative stress (cytochrome c and SOD expression) in patients with pulmonary arterial hypertension. The results suggest that PGC-1α likely modulates by oxidative mechanisms at the mitochondrial level of patients with pulmonary hypertension and could be used as a biomarker since it is not expressed healthy subjects.

In conclusion, the growing body of evidence suggests that the paradoxical increase in ROS generation is at the heart of the pathologic mechanisms associated with the hypoxic feature. In this respect, we are convinced that the study of the underlying mechanisms increasing oxidative stress in the pathologies associated with hypoxia will be essential to develop the future therapies/treatments of these diseases.

Acknowledgments

This special issue would not be possible without the great efforts of the authors and the reviewers. In this regard, we would like to thank all these people that took part in the achievement of this issue.

Vincent Pialoux
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Research Article

Blueberry Extracts Protect Testis from Hypobaric Hypoxia Induced Oxidative Stress in Rats

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Exposure to hypobaric hypoxia causes oxidative damage to male rat reproductive function. The aim of this study was to evaluate the protective effect of a blueberry extract (BB-4) in testis of rats exposed to hypobaric hypoxia. Morphometric analysis, cellular DNA fragmentation, glutathione reductase (GR), and superoxide dismutase (SOD) activities were evaluated. Our results showed that supplementation of BB-4 reduced lipid peroxidation, decreased apoptosis, and increased GR and SOD activities in rat testis under hypobaric hypoxia conditions ($P < 0.05$). Therefore, this study demonstrates that blueberry extract significantly reduced the harmful effects of oxidative stress caused by hypobaric hypoxia in rat testis by affecting glutathione reductase and superoxide dismutase activities.

1. Introduction

Berries are a recognized source of antioxidants since they contain phytochemicals, nonenzymatic factors of plant origin that significantly benefit health [1, 2]. Such extracts have proven to be effective in preventing the effects of oxidative stress under different pathological conditions [3–6]. Among the different species, there is a group classified as blueberries that have a dark color due to anthocyanins and polyphenols as principal pigments with antioxidant activities [3]. Phytochemicals have been demonstrated to be powerful inhibitors of lipid peroxidation when compared to other classic antioxidants [3, 7], and the protective effect of polyphenols against oxidative damage seems to be via glutathione system [8].

The enzymatic mechanism against oxidative stress is made of free radical scavengers like superoxide dismutase (SOD), catalase (CAT), and the glutathione-dependent enzymes such as glutathione peroxidase (GPx), glutathione S-transferase (GSH), and Glutathione reductase (GR) [9]. GR and enzymatic antioxidant mechanisms play an essential role in preventing oxidative damage in cells and tissues [10].

We have previously described that hypobaric hypoxia induced oxidative damage, decreased glutathione reductase activity and ascorbic acid, and had a protective role against oxidative stress [11]. The effect of a reduced spermatogenesis under hypobaric hypoxia [12] is accompanied by an increased vascularization and reactive oxygen species (ROS) in the testis [13, 14]. These vascular changes are induced by ROS via inhibition of prolyl hydroxylase domain (PHD) proteins [11]. The activity of PHD seems to be restored by a supplement of ascorbic acid [15] making it possible to generate strategies for administering antioxidants to prevent the effects of hypobaric hypoxia as previously suggested [14, 16, 17].

Previously, It has been demonstrated that enriched blueberries reduced the adverse effects of oxidative stress in rat neuron cell lines and brain tissues [18, 19]. Such extract has shown to cross the blood-brain barrier [19, 20]. Brain

homeostasis and spermatogenesis depend on blood-to-brain and blood-to-germ cells transport of metabolites and substances [21], therefore it was of interest to determine whether the protective effect can be induced in rat testis *in vivo* model. The aim of this work was to evaluate the protective effect of a blueberry-enriched polyphenol extract (BB-4) against oxidative stress in rat testis exposed to hypobaric hypoxia.

2. Materials and Methods

2.1. Experimental Design. Ten-week-old Sprague Dawley rats (*Rattus norvegicus* specie) were divided into six groups (5 rats per group): (1) normobaric conditions (Nx); (2) Nx plus administration of physiological solution (PS); (3) Nx plus blueberry extract (BB-4), rich in polyphenols, (BB-4); (4) hypobaric hypoxia (HH); (5) HH plus PS and (6) HH + BB-4. Rats were housed under a 12 hours of Light: 12 hours of Dark cycles and the humidity was 61 ± 9%. BB-4 was administered intraperitoneally (10 mg dry extract/kg of body weight) or with physiological solution (1 mL of NaCl 9 mg/mL) at 96-hour intervals. The selection of the dose and route of administration of BB-4 was based on previous work reported [11, 14] where the protective effect of compounds did not affect the liver as analyzed by the presence of transaminases in the blood of animals. Groups 4, 5, and 6 were exposed to HH conditions for 96 hours in a hypobaric chamber (428 tor; pO2: 89.6 mmHg) for a period of 32 days. The desired pressure inside the hypobaric chamber was achieved by pressure changes simulating altitude increases of 150 meters per minute. The animals in the Nx groups were lodged in the same room as the HH (22°C, 15 g of pellet meals per day and 250 mL of water per rat). All procedures complied with the principles of animal care outlined by the National Society Laboratory and the Medical Research, and the Guide for the Care and Use of Laboratory Animals (Institute of Animal Laboratory Resources, 1996).

2.2. Blueberry Extracts. Six polyphenol-enriched blueberry extracts were obtained from fresh blueberries, locally harvested, using different solvents (with increasing polarity), and denominated BB-1–6, depending on the solvents employed (chloroform, acetone/water, ethanol, ethanol/water, water/acetone, or methanol). The most active extract was obtained with ethanol/water and was denominated BB-4. To obtain the enriched polyphenol extracts (BB), we used an Amberlite XAD-7 adsorber resin (Merck, Darmstadt, Germany) and an Amberlite XAD-2 adsorber resin (Supelpack 2; Sigma-Aldrich). The extract obtained had 1.5% polyphenols for each 100 g of fresh fruit, where the main components were rutin (0.34%) and isoquercetin (0.42%). The BB-4 extract was shown to be the most active in the preliminary activity screening test and it was then characterized to determine its polyphenol composition through HPLC techniques. The final BB-4 extract was dried and diluted in dimethylsulfoxide (DMSO) to a final concentration of 810 mg/liter. Different dilutions from 1 : 10 to 1 : 100,000 were prepared daily in external solution containing 5 mM $CaCl_2$, 100 mM NaCl, 45 mM tetraethylammonium chloride (TEACl), 10 mM HEPES, 5.5 mM KCl, and 10 mM glucose [18].

2.3. Organ Collection. The animals were weighed and sacrificed by cervical dislocation. The testes were removed and placed in a saline buffer PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM sodium phosphate monobasic, pH 7.2) (Sigma Chemicals, St Louis, MO, USA) for further studies.

2.4. Preparation of Tissue Homogenate and Protein Assay. The 100% of testis were completely homogenized in 0.5 mL of extraction buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, Tween-20 0.1% v/v pH 7.4, 100 μg/mL PMSF (Sigma Chemicals, St Louis, MO, USA) with a Potter glass homogenizer (Glass-Col K4424, CA, USA) at 50 rpm. Then the samples were centrifuged for 30 minutes at 4°C. The protein concentration was determined on the supernatant using the Coomassie blue method [22].

2.5. Glutathione Reductase Expression Determined by Western Blot (SDS/PAGE). Aliquots of tissue homogenate of testis containing equal concentration of proteins, 50 μg, were electrophoresed (120 mV) in a 12% SDS/PAGE gel as previously described by Farias et al. [11], using a primary anti-rat glutathione reductase antibody against rabbit (1 : 500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a secondary anti-rabbit antibody (Jackson Immune Research Laboratories, PA, USA). β-tubulin was used as a loading control in all Western blot assays. The bands obtained were analyzed with Image J Software (http://rsbweb.nih.gov/ij/download.html) and the integrated density values of the glutathione reductase bands were normalized by dividing by the value of the loading control band.

2.6. Histological Procedures. Testis from each animal was weighed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 hours at room temperature. The testicles were embedded in paraffin after dehydration in ascending alcohol concentrations. Five sections were cut from the equatorial zone toward the testicular apex and mounted on glass slides and stained with hematoxylin-eosin sections.

2.7. Determination of Cellular DNA Fragmentation. In order to detect apoptosis by DNA fragmentation, an *in situ* Oligo Ligation method (ApoTag ISOL, Q-BIOgene, UK) was carried out as described by Lesauskaite et al. [23]. This method is based upon the specificity of the enzyme T4 DNA ligase [24]. In these experiments, we utilized five 5 μm tissue sections that were obtained from rat testicles from the equatorial zone toward the testicular apex. The distance between the sections corresponded to 120 μm. This procedure was repeated for every condition tested.

2.8. Determination of Lipid Peroxidation in the Testis. The protocol for the thiobarbituric acid (TBA) (Sigma Chemicals, St Louis, MO, USA) assay was carried out as described by Draper and Hadley [25]. 5 mg of tissue homogenates were centrifuged at 10 000 g for 10 min at 4°C to sediment mitochondria and cell debris. The sediments were suspended in PBS pH 7.4, mixed with BHT-TCA solution (1% w/v BHT dissolved in 20% w/v TCA), and centrifuged at 1000 g for 5 min. Supernatant was then mixed with 0.5 N HCl and 120 mM TBA in 26 mM Tris and heated in a water bath at 80°C for 10 min. After cooling, the absorbance of the resulting chromophore was determined at 532 nm using a OPTIZEN UV-Visible spectrophotometer (3220UV) and MDA production was determined by using an extinction coefficient of $1.56 \times 10^5\,M^{-1}\,cm^{-1}$. The results were expressed as nmol of MDA equivalents/mg tissue.

2.9. Determination of Glutathione Reductase Activity in the Testis. To measure glutathione reductase activity, homogenates were thawed at room temperature and centrifuged at 700 ×g for 10 min, after which 20 μl of supernatant was added to quartz cuvettes containing a fresh solution of 0.44 mM GSSG, 0.30 M EDTA, in 0.1 M phosphate buffer—pH 7.0—and 0.036 M NADPH was added just before the enzymatic determination as the starting reagent. The assay was run at 340 nm for 4 min with absorbance readings taken every 30 s. Glutathione reductase activity was estimated using NADPH extinction coefficient of $6.2\,mM^{-1} \cdot cm^{-1}$ and expressed as U/mg of protein [11, 26].

2.10. Determination of Superoxide Dismutase Activity in the Testis. Superoxide dismutase activity was measured by the autoxidation of pyrogallol method as described by S. Marklund and G. Marklund [27]. The activity of superoxide dismutase was assayed in 1000 ×g supernatants of testis homogenates. One unit of superoxide dismutase was defined as the amount of protein that caused 50% pyrogallol autoxidation inhibition. Superoxide dismutase activity was expressed as U/mg protein.

2.11. Evaluation of Hematocrit, Body Weight, and Testicular Mass Relative to Body Weight. Blood samples were obtained with a needle from the left ventricle of the rat once sacrificed. The percentage of hematocrit was determined by centrifugation of the capillary tube with heparinized blood in a microhematocrit centrifuge (IEC Model MB, GSR Technical Sales, Canada). The animals were weighed and the testicular mass relative to body weight was determinate at the end of 32 days: (Testicular mass/body weight) $*$ 100 = (%).

2.12. Statistical Analysis. The results were analyzed as previously described by Farias et al. [11] by the two-way ANOVA in order to determine the presence of a significant interaction between the environmental factors (sea level and high altitude) and the injection factors (without injection, vehicle and dose), making it possible to determine whether the treatment with BB-4 under IHH produces any significant effect on the variables different to that produced in Nx. Also, the two-way ANOVA enabled to determine whether at least one level of each factor (environmental or injection) affected the results and to determine whether the changes in the barometric pressure or the administration of BB-4 produced any significant effect on the results. The statistical significance was established to $P < 0.05$ for all analyses and a Bonferroni test was performed to compare treatments. Data were analyzed using the Graph Pad Prism Software v4.0 (San Diego, CA, USA). The results are presented in graphs with standard deviation of the mean (SD).

3. Results

The effect of hypobaric hypoxia exposure on testicular mass, testicular mass relative to body weight, diameter of seminiferous tubule, and height of epithelium was reversed with treatment with BB-4 ($P < 0.05$). Indeed, all these parameters came back to similar levels to those obtained in Nx (Figures 1(a), 1(b), and 1(c); Table 1). The hypoxia hypobaric condition induced apoptotic DNA fragmentation in spermatogenic cells in rats (Figure 1(d); $P < 0.05$). However, in rats subjected to hypobaric hypoxia and treated with BB-4, the apoptotic index significantly decreased ($P < 0.05$). On the other hand, lipid peroxidation (TBARS) was significantly higher ($P < 0.05$) under hypobaric hypoxia as compared to normoxic conditions in the testis as shown in Figure 2(a). The blueberry extract (BB-4) did not affect rats exposed under normoxia; however, this substance reduced lipid peroxidation in treated rats with the extract ($P < 0.05$). BB-4 seemed to protect the testis only under hypobaric hypoxic conditions.

There was a significant increase in glutathione reductase and superoxide dismutase activities under hypobaric hypoxia in comparison to normoxic groups ($P < 0.05$) as shown in Figures 2(b) and 2(c). BB-4 seemed to protect the testis under hypobaric hypoxic conditions when the enzymes SOD and GR increased.The specific activities of these enzymes significantly increased ($P < 0.05$) in rats treated with blueberry extract and subjected to hypobaric hypoxia ($P < 0.05$). There were no significant differences in glutathione reductase protein expression under hypoxia in comparison to normoxic groups ($P > 0.05$), as observed in Figure 2(d). The hematocrit was significantly greater ($P < 0.05$) in groups subjected to hypobaric conditions when compared to normoxic groups (Figure 3(b)). The blueberry extract did not have any effect on the polycythemia that usually characterizes exposure to hypobaric hypoxia as a compensatory mechanism to the drop in arterial pressure of oxygen. Our results showed a significant decrease ($P < 0.05$) in body weight of hypobaric hypoxia groups in comparison to normoxic groups (Figure 3(a)). However, blueberry extract treatment did not affect this parameter under any environmental conditions.

4. Discussion

These results showed that the levels of lipid peroxidation in all groups subjected to hypobaric hypoxia induced

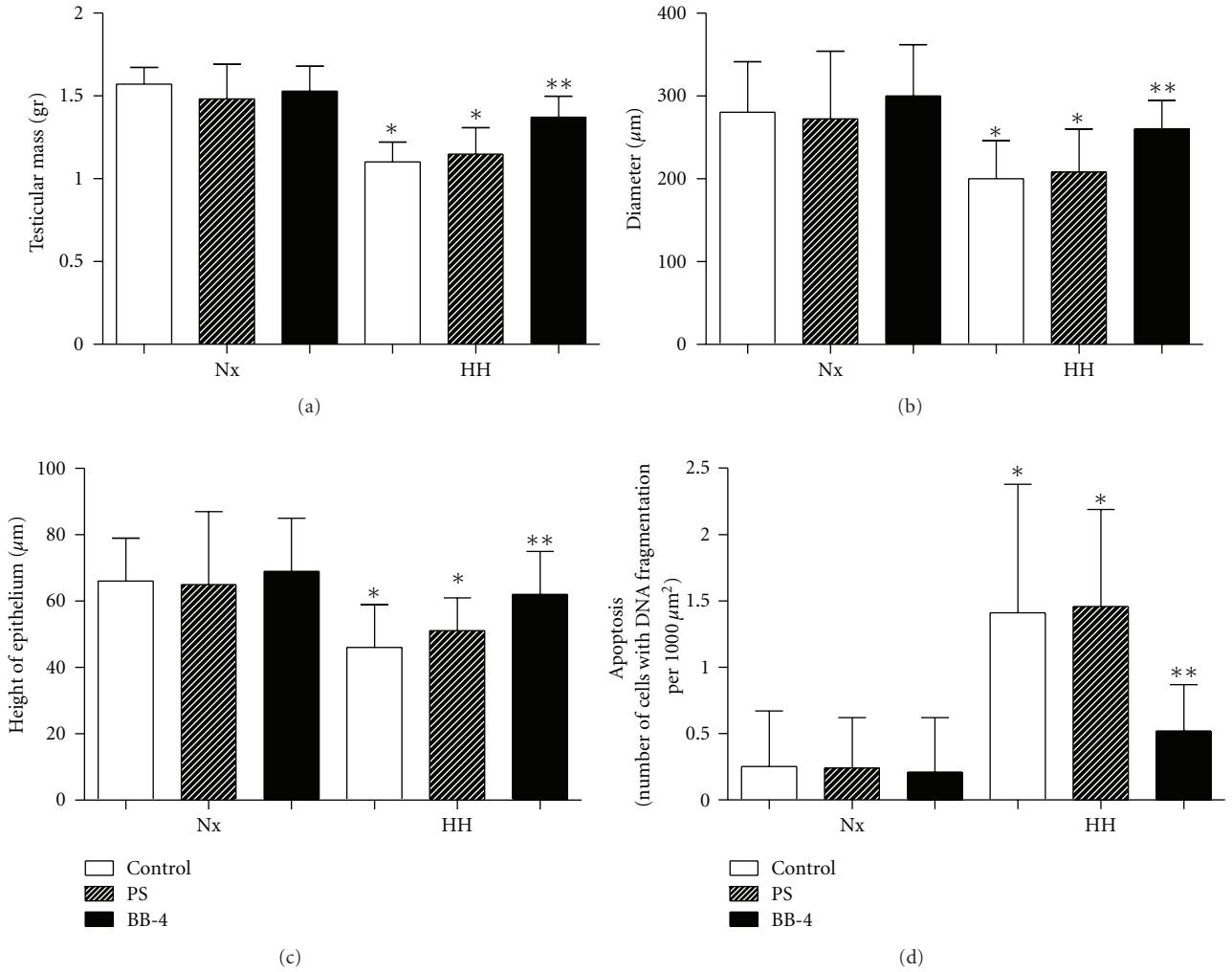

Figure 1: Effect of intermittent hypobaric hypoxia and BB-4 on (a) testicular mass (gr), (b) diameter (μm), epithelium height, (c) and (d) apoptotic index (number of cells with fragmented DNA per 1000 μm^2). Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. Experiment: 32-day treatment. Bars indicate the mean ± SD ($n = 5$). $^*P < 0.05$ (HH versus Nx control); $^{**}P < 0.05$ (HH + BB-4 versus HH control).

Table 1: Testicular mass relative to body weight (%).

Nx	Nx + PS	Nx + BB-4	HH	HH + PS	HH + BB-4
0.44 ± 0.02	0.41 ± 0.03	0.43 ± 0.02	0.40 ± 0.01*	0.40 ± 0.04*	0.48 ± 0.03**

Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. (Testicular mass/body weight) ∗ 100 = (%). Mean ± SD. $^*P < 0.05$ (HH versus Nx control); $^{**}P < 0.05$ (HH + BB-4 versus HH control).

oxidative stress causing a decrease in testicular mass, arrest of spermatogenesis, and an increase in apoptosis. In previous studies, we reported that hypobaric hypoxia caused oxidative damage and histological changes in testis [11–13].

The production of reactive oxygen species at the testicular level can be especially accentuated by the presence of a basal hypoxic microenvironment and abundance of polyunsaturated fatty acids [28, 29]. On the other hand, the activities of glutathione reductase and superoxide dismutase were significantly reduced. We previously found that enzyme expression did not change in rat testis under hypobaric hypoxia [11].

Several studies have demonstrated the beneficial effects of blueberries against oxidative stress. Blueberries have the ability to neutralize oxygen-containing free radicals [30]. Phenolic compounds with strong antioxidant properties are found in the diet and can prevent oxidative damage as a result of their ability to scavenge reactive oxygen species [31]. It has been reported that blueberries have protective effects against oxidative damage in animals [19, 32]. Animals exposed to

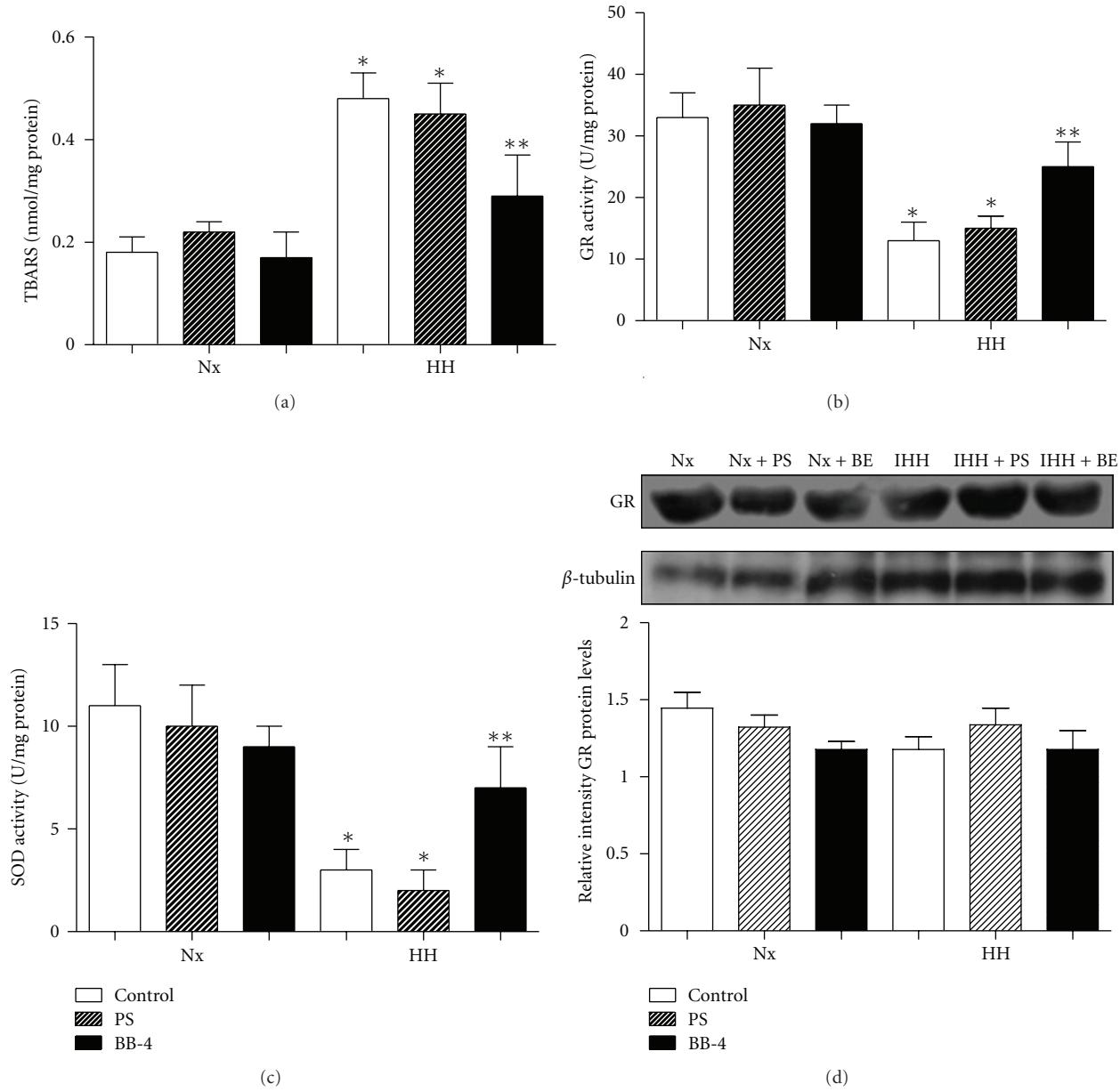

Figure 2: Effect of intermittent hypobaric hypoxia and BB-4 on (a) lipid peroxidation (TBARS) (nmol/mg protein), (b) glutathione reductase (GR) activity (U/mg protein), (c) superoxide dismutase (SOD) activity, and (d) glutathione reductase protein expression. Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. Experiment: 32-day treatment. Bars indicate the mean ± SD ($n = 5$). $^{*}P < 0.05$ (HH versus Nx control); $^{**}P < 0.05$ (HH+BB-4 versus HH control).

hypobaric hypoxia and treated with blueberry extract (BB-4) showed a significant decrease in lipid peroxidation in rat testis reaching levels similar to normoxic condition. On the other hand, blueberry extract prevented the effects of hypobaric hypoxia on testicular mass, arrest of spermatogenesis, and apoptosis. The effect of blueberry extract on spermatogenic cells can be attributed to a possible passage through the hematotesticular barrier and thus protecting this tissue from oxidative stress generated by hypobaric hypoxia. Our results indicated that there were no changes in GR expression. However, the enzyme activity was significantly restored in animals subjected to hypobaric hypoxia and treated with blueberry extract suggesting that these compounds could activate the powerful endogenous antioxidant defenses by chemically reducing oxidized glutathione [33].

Hypobaric hypoxia caused a significant loss in body weight. The effect of hypoxia on body weight has been described by various authors who have indicated that mammals exposed to different hypoxia exposure experienced weight loss, dehydration, fat loss, and muscular mass reduction [34–36]. The blueberry extract did not have a protective effect on body weight. Our data suggested that blueberry

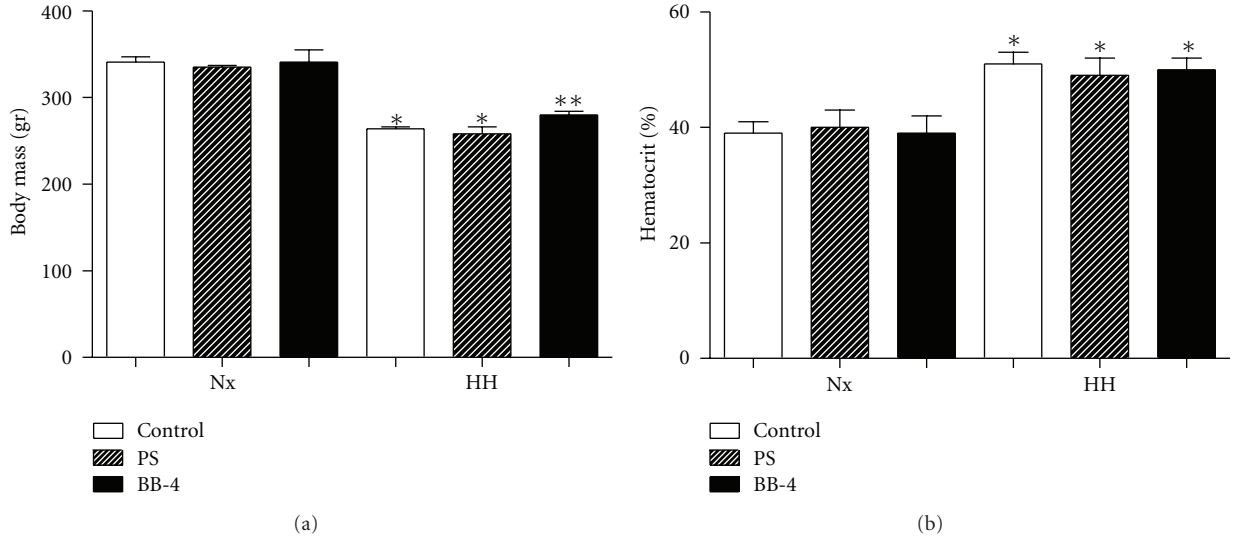

Figure 3: Effect of intermittent hypobaric hypoxia and BB-4 on (a) body weight and (b) hematocrit (%). Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. Experiment: 32-day treatment. Bars indicate the mean ± SD ($n = 5$). $^{*}P < 0.05$ (HH versus Nx control); $^{**}P < 0.05$ (HH+BB-4 versus HH control).

extract may blunt the oxidative stress induced by hypobaric hypoxia as showed by the decreased lipid peroxidation and the increased glutathione reductase and superoxide dismutase activities with BB-4 compared to control. The dose of BB-4 did not affect polycythemia, a characteristic of exposure to hypobaric hypoxia as a compensatory mechanism to the drop in arterial PO_2. A significant increase in erythrocytes was observed in animals exposed to intermittent hypobaric hypoxia, which indicated that BB-E had no effect on erythropoietin; therefore, it did not affect one of the mechanisms of acclimatization to high altitudes [37].

These results corroborate previous studies demonstrating the beneficial effects of polyphenols present in natural and enriched foods [18]. Blueberry extract presented a protective effect against oxidative stress induced by hypobaric hypoxia by recovering glutathione reductase and superoxide dismutase activities. It can be concluded that extracts of natural origin can be effective in the prevention of oxidative stress induced by hypobaric hypoxia, and it opens the possibility of generating additional health benefits to people who live under conditions of oxidative stress.

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Review Article

Hypoxia-Induced Oxidative Stress in Ischemic Retinopathy

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Oxidative stress plays a crucial role in the pathogenesis of retinal ischemia/hypoxia, a complication of ocular diseases such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP). Oxidative stress refers to the imbalance between the production of reactive oxygen species (ROS) and the ability to scavenge these ROS by endogenous antioxidative systems. Free radicals and ROS are implicated in the irreversible damage to cell membrane, DNA, and other cellular structures by oxidizing lipids, proteins, and nucleic acids. Anti-oxidants that can inhibit the oxidative processes can protect retinal cells from ischemic/hypoxic insults. In particular, treatment using anti-oxidants such as vitamin E and lutein, inhibition of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) or related signaling pathways, and administration of catalase and superoxide dismutase (SOD) are possible therapeutic regimens for DR, ROP, and other retinal ischemic diseases. The role of oxidative stress in the pathogenesis of DR and ROP as well as the underlying mechanisms involved in the hypoxia/ischemia-induced oxidative damage is discussed. The information provided will be beneficial in understanding the underlying mechanisms involved in the pathogenesis of the diseases as well as in developing effective therapeutic interventions to treat oxidative stress-induced damages.

1. Introduction

The retina is highly susceptible to oxidative damage by reactive oxygen species (ROS). Besides having the highest oxygen consumption in the body [1], the retina is also prone to photo-oxidation due to its constant exposure to incoming light. The high oxygen consumption and endless light exposure in the retina may in turn generate ROS. Moreover, the high lipid content in the retina (due to abundant polyunsaturated fatty acids in the photoreceptor outer segment) makes it prone to lipid peroxidation. During pathological conditions such as retinal ischemia, the imbalance between the production of ROS and the ability to scavenge these ROS by endogenous antioxidant systems is exaggerated. ROS triggers several signaling pathways, affects DNA and lipids inside the cell, and subsequently leads to cell death. Antioxidants that can inhibit or prevent the oxidative processes can protect retinal cells from ischemic damage.

2. Sources of Oxidants

A free radical is an atom, a molecule, or an ion having unpaired electrons. Due to the presence of the "free" electron in the outer shell, free radical is chemically very unstable and reactive. In order to achieve stability, free radical will participate in further reduction and oxidation reactions. Therefore, production of one free radical leads to further radical formation via sequential chain reactions [2]. Free radicals that are derived from oxygen are ROS, being one of the major contributors of oxidative stress. They include superoxide anion (O_2^-), perhydroxyl radical (also known as hydroperoxyl radical, HO_2), and hydroxyl radical (OH). Superoxide is formed by either the enzymatic reduction of oxygen by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) or nonenzymatic reaction of mitochondrial respiration [3]. It is converted into hydrogen peroxide (H_2O_2) enzymatically by superoxide dismutase (SOD) or

nonenzymatically to H_2O_2 and singlet oxygen [3, 4]. Singlet oxygen is a powerful oxidant and is able to oxidize lipids, proteins and nucleic acids [5]. Perhydroxyl radical is the protonated form of superoxide, acting as an oxidant and reacts with polyunsaturated fatty acids in the membrane lipid bilayer, thereby initiating lipid peroxidation. Hydroxyl radical is another reactive radical which can be formed either from the reaction of singlet oxygen and H_2O_2 by the Haber-Weiss reaction [6] or from the interaction between H_2O_2 and reduced transition metals such as ferrous ions by the Fenton reaction [3, 6, 7]. Generation of superoxide, singlet oxygen and hydroxyl radical causes detrimental effects on the physiological states of cells by cleaving covalent bonding in protein and carbohydrates, leading to lipid peroxidation and compromising the integrity of cell membrane [3, 6, 7].

Another free radical, nitric oxide (NO) is generated from the oxidation of the guanidine group of L-arginine catalyzed by the enzyme nitric oxide synthase (NOS). NO is in fact a signaling molecule. Moreover, NO can react with superoxide to from a strong oxidant, peroxynitrite (ONOO), one of the reactive nitrogen species (RNS). Similar to ROS, excessive production of RNS (nitrosative stress) results in nitrosylation, causing deleterious effects such as lipid peroxidation, DNA damage and SOD inactivation [8]. Therefore, the toxic effects of ONOO and NO should also be taken into account in developing the antioxidative strategy [9].

3. Anti-Oxidative Mechanisms and Oxidative Stress

In physiological condition, ROS and RNS are products of normal cellular metabolism. ROS can be efficiently scavenged by the intrinsic antioxidant defense mechanisms. Manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/Zn SOD), catalase, and glutathione peroxidase (GPx) are members of the enzymatic antioxidants while ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), and β-carotene are nonenzymatic antioxidants. For example, MnSOD and Cu/Zn SOD convert superoxide to H_2O_2, which is then efficiently changed to water by GPx or catalase [10, 11]. On the other hand, vitamin E, vitamin C and β-carotene act as radical scavengers. Vitamin E also reduces and removes lipid peroxyl radical, a product of the reaction between lipid radical (a result of electron extraction from polyunsaturated fatty acid by hydroxyl radical) with molecular oxygen and an initiator of lipid peroxidation within the cell membrane. Subsequently, vitamin E is regenerated by vitamin C. Therefore, both vitamin E and vitamin C help to cease the lipid peroxidation chain reactions involving lipid peroxyl radicals [12]. GSH, a major cellular redox buffer, can directly scavenge hydroxyl radical and singlet oxygen. Through the action of GPx, GSH can also remove H_2O_2 and lipid peroxides. Moreover, GSH helps to regenerate both vitamin E and vitamin C back to their active forms. Normally, a balance is maintained between the levels of oxidants and antioxidants, which is essential for survival.

During ischemic injury, there is overproduction of free radicals that in turn leads to further production of radicals through chain reactions [10, 12]. The imbalance between the overproduction and scavenge of ROS/RNS leads to oxidative stress/nitrosative stress. The excess ROS can damage cellular lipids, proteins, or DNA and in turn inhibit their normal function. ROS also disturbs normal functioning of mitochondria by opening the mitochondrial membrane permeability transition pores (PTPs), leading to energy uncoupling and further ROS production [13]. This also induces the release of cytochrome c, leading to apoptosis [10, 14, 15]. In addition, superoxide reacts with NO to form ONOO anion and in turn causes oxidation, lipid peroxidation, and subsequently cellular damage [8]. Accumulation of ONOO and free radicals in the nucleus also leads to DNA damage [9]. Indeed, an increased level of nitrotyrosine, the nitration product of ONOO, is observed after retinal ischemia/reperfusion (I/R) injury and is attributed to cell death [16, 17].

Retinal ischemia is a common feature in ischemic retinopathy such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP). In both ocular diseases, there is initially an impairment in the normal retinal blood supply while the subsequent formation of abnormal new blood vessels (retinal neovascularization) further worsens the condition. The presence of ischemia and consequently oxidative stress makes the already vulnerable retina more prone to oxidative damage.

4. Oxidative Stress and the Pathogenesis of Diabetic Retinopathy

DR is one of the major causes of blindness worldwide. Most importantly, all patients with diabetes mellitus are at risk. The longer the patient has diabetes, the higher the chance of developing DR. As the total number of people with diabetes, estimated to be 171 million worldwide in 2000 [18] is rising, DR causes enormous economic and social burden to the society. In particular, DR affects working-age adults. This poses extra need and effort in treating the disease. Despite extensive research, the promising therapeutics has yet to come. Kowluru and Zhong have recently published an excellent review in antioxidant therapy for age-related macular degeneration (AMD) and DR, providing evidence on the positive potential of antioxidant therapy clinical trials in DR treatment [18] (Figure 1).

Basement membrane thickening, pericytes loss, microaneurysms, blood-retinal barrier breakdown, and neovascularization are pathological features of DR [19]. Microvascular complication is a manifestation of DR which leads to retinal ischemia/hypoxia and exacerbates the condition [20, 21]. Four major mechanisms are involved in development of pathologies in DR: increased polyol pathway flux, increased formation of advanced glycation end-products (AGEs), activation of protein kinase C (PKC) isoforms, and increased hexosamine pathway flux [22].

Increasing body of evidence shows that these metabolic mechanisms are associated with excess production of ROS and depletion of antioxidants in DR [19]. Firstly, aldose

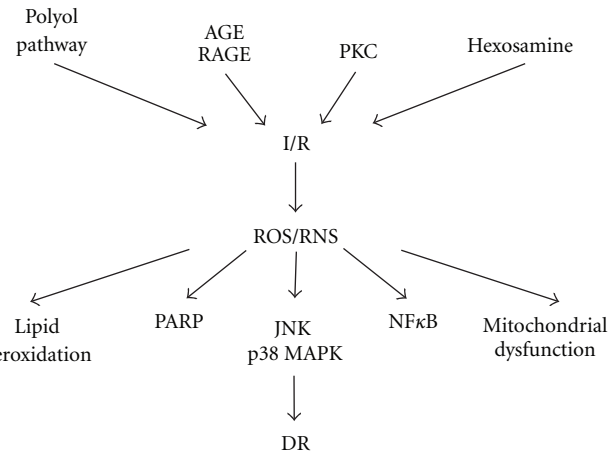

Figure 1: Schematic showing the relationship between oxidative stress and DR. The four major mechanisms involved in DR: increased polyol pathway flux, increased AGE formation, activation of PKC pathway, and increased hexosamine pathway flux results in I/R and increased production of ROS/RNS, thereby changing the levels of JNK/p38 MAPK, PARP, and NFκB, increasing lipid peroxidation as well as leading to mitochondrial dysfunction.

reductase (AR) is the first and rate-limiting enzyme in the polyol pathway. In a hyperglycemic condition, AR reduces glucose to sorbitol, consuming the cofactor NADPH. Sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase. As NADPH is used for generating the intracellular antioxidant GSH, a reduction in the availability of NADPH exacerbates intracellular oxidative stress. The increase in enzymatic activity of AR during hyperglycemia further worsens the situation of DR [22, 23]. On the other hand, inhibition of AR activity, either by specific inhibitor or gene deletion, may have a therapeutic role in preventing the progression of diabetic retinopathy [24–26] and ischemia-related ocular diseases [21, 27, 28]. Application of the AR inhibitor, fidarestat, is beneficial in decreasing the concentrations of sorbitol and fructose and accumulation of leukocytes in diabetic retina [24]. Secondly, AGEs and its receptor, RAGE, can also cause oxidative stress. AGEs induce oxidative damage by overproduction of superoxide in neuronal culture through the activation of NADPH oxidase in a PKCδ-dependent manner [29–31]. Increased intracellular formation of AGE and RAGE activates a cascade of signaling proteins and attributes to intracellular redox imbalance, which leads to overproduction of ROS [29–31]. On the other hand, inhibition of AGE formation and suppression of the RAGE-mediated downstream pathway have been suggested to be the feasible therapeutic strategies for DR [32, 33]. In addition, hyperglycemia itself leads to intracellular accumulation of glyceraldehyde-3 phosphate. Glyceraldehyde-3 phosphate further induces the production of ROS and leads to subsequent accumulation of poly(ADP-ribose) polymerase-1 (PARP), which triggers the activation of the PKC signaling pathway and increases AGE formation [34]. Indeed, activation of PKC signaling pathway and overproduction of ROS by the mitochondria electron transport chain are the key factors in the pathogenesis of DR [19, 23].

Oxidative stress causes the impairment of mitochondrial function by damaging the inner membrane of mitochondria, which leads to imbalance in the electron transport chain and consequently leads to further overproduction of superoxide, ONOO and hydroxyl radicals [35, 36]. Studies have shown that ROS also impairs mitochondrial function and transport machinery by damaging the mitochondrial DNA and increases apoptosis of retinal capillary cells during DR [37–39]. Increased ROS levels also trigger the release of cytochrome c, which in turn damages the mitochondrial membrane potential and initiate apoptosis by the activation of caspase-9 and caspase-3 [40–43]. Apoptosis in DR is also regulated by the family of Bcl-2 signaling proteins [43–45].

Several signaling pathways are involved in the pathogenesis of DR. Mitogen-activated protein kinase (MAPK) has been shown to play a role in hyperglycemia-induced cell death in DR [46]. In a cell culture model of hyperglycemia and hypoxia, phosphorylation of c-Jun N-terminal kinases (JNK) and p38 MAPK is induced, leading to overproduction of ROS and disruption of tight junctions in ARPE-19 cells [47]. In streptozotocin-induced diabetic mice, activation of cannabinoid-1 receptor contributes to DR by increasing MAPK activation, oxidative stress, and inflammatory signaling [48]. In addition, PARP and nuclear factor-kappa B (NFκB) are also downstream effectors of oxidative stress. PARP is a DNA nick-sensor enzyme that is activated by DNA single-strand breaks. It is involved in DNA repair, gene transcription, cell death, and apoptosis and acts as a coactivator in NFκB signaling pathway [34, 49, 50]. Increased levels of oxidative and nitrosative stress induce DNA single-strand break and activates PARP. This in turn inhibits the activity of GAPDH by poly(ADP-ribosylation) and consequently leads to endothelial dysfunction in diabetic conditions [34]. Therefore, inhibition of PARP could decrease the activation of PKC isoforms, hexosamine pathway flux and AGE formation induced by hyperglycemia [34]. As oxidative stress activates PARP, which in turn initiates the activation NFκB, application of PARP inhibitor could successfully inhibit hyperglycemia-activated NFκB signaling pathway in endothelia cell culture [51]. Also, inhibition of PARP or NFκB reduces the hyperglycemia-induced cell death in retinal endothelial cells [50]. NFκB also plays a role in apoptosis by triggering the Notch-1/Akt and PI3k/Akt signaling pathways. Increased activation of PARP, cleaved caspase-3 as well as reduced expression of Notch1 and pAkt have been observed, which consequently leads to apoptosis in hyperglycemic retina [49].

5. Oxidative Stress and the Pathogenesis of Retinopathy of Prematurity (ROP)

Similar to DR, retinal ischemia/hypoxia is a feature in ROP, which is a leading cause of blindness in children worldwide. Retinopathy of prematurity (ROP) was first described as a disease of prematurity [52, 53] and contributed to 50% of infant blindness in 1950. The percentage dropped to 4% in 1965 after identification of supplemental oxygen use as a

risk factor. Yet, with improved neonatal care and increased survival of very low birth weight preterm infants the incidence of ROP increased in recent years. ROP is now a major cause of childhood blindness in developed and developing countries. Latest estimates from National Eye Institute showed that 1,100–1,500 infants (~5% infants ≤1.25 kg at birth, <31 weeks of gestation) develop severe ROP that requires treatment. Despite treatment, 400–600 infants (40% with ROP, NIH figure) become legally blind. The resulting long-term disability and severely affected quality of life in ROP patients pose intense burden on the healthcare system worldwide as the population ages, making ROP a major public health issue.

ROP is an ocular disease characterized by vascular abnormalities induced by two phases of pathological changes. The first phase starts with the cessation of retinal vessel growth after premature birth. As premature infants are exposed to high oxygen inside the incubator after birth, a condition of relative hyperoxia happens, leading to downregulation of vascular endothelial growth factor (VEGF) and a subsequent regression of developed retinal vessels. After the cessation of oxygen therapy, infants are returned to normal oxygen tension and a condition of relative hypoxia occurs. In addition, the metabolic demand of the relatively vascular-depleted retina is now higher as the infant grows. Therefore, the increased demand for oxygen poses a hypoxic injury to the retina. The relatively hypoxic condition of the retina triggers the abnormal proliferation of vessels and leads to neovascularization; this is the second phase of ROP progression [54]. Hypoxia-induced oxidative stress therefore, plays a crucial role in the pathogenesis of ROP (Figure 2).

It has been shown that the production of enzymatic and nonenzymatic antioxidants such as SOD, catalase, GPx, vitamin C, and vitamin E increases dramatically in the late stage of gestation [55, 56]. Yet, premature infants are born before term; they are therefore, more susceptible to oxidative stress-induced damage due to the inadequate levels of antioxidants in premature birth and the inability of antioxidant synthesis during the high-oxygen intensive care period [54]. Indeed, the levels of 8-hydroxy 2-deoxyguanosine (8-OHdG), an indicator of oxidative stress, in leukocyte and urine in ROP infants are significant higher than the full-term infants, confirming the presence of oxidative stress in ROP patients [57].

During the two phases of ROP, the retina is subjected to fluctuating oxygen tensions, resulting in retinal hypoxia that triggers the overproduction of ROS, which activates NADPH oxidase and attributes to intravitreal neovascularization by the activation of signaling pathway such as JAK/STAT [58, 59]. On the other hand, inhibition of NADPH oxidase and JAK/STAT signaling pathway reduce the level of cleaved caspase-3 and hence, apoptosis and neovascularization in ROP retina [58, 59]. Increase intake of omega-3-polyunsaturated fatty acids has been shown to reduce the vaso-obliteration and neovascularization by suppressing the TNF-α in ROP retina [60]. Antioxidants such as vitamin E could also decrease the vaso-obliteration

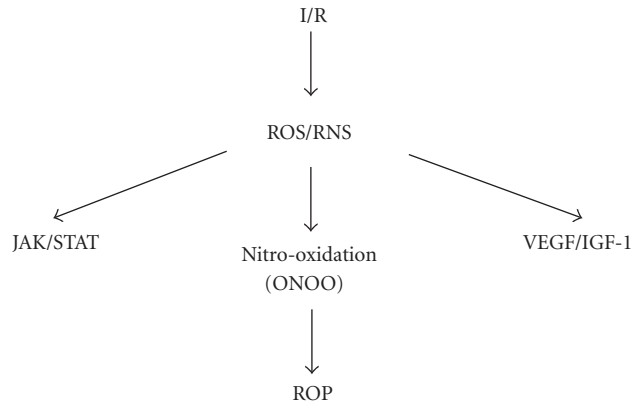

Figure 2: Schematic showing the relationship of I/R, oxidative stress, and ROP. I/R causes overproduction of ROS/RNS. The resulting changes in levels of JAK/STAT, nitro-oxidation, and VEGF/IGF-1 contribute to pathogenesis of ROP.

in ROP retina by its ROS scavenging property [61]. All this evidence points to the importance of oxidative stress in the pathogenesis of ROP.

Nitro-oxidation also contributes to the pathogenesis of ROP. Increased level of NOS, which contributes to the increase of NO production, is observed in neonatal retina exposed to hypoxia [62]. In an animal model of ROP, increased formation of ONOO and apoptosis of endothelial cell were seen in association with increased tyrosine nitration of PI3K, cleaved caspase-3, activation of p38 MAPK signaling pathway, and decreased Akt phosphorylation. However, blocking tyrosine nitration of PI3K with epicatechin or N-acetylcysteine reverses the nitro-oxidation-induced pathogenesis [63]. In addition, application of NOS inhibitor or gene deletion of endothelial NOS effectively reduces the severity of ROP in mice, indicating the crucial role of nitro-oxidative stress in ROP [64]. Moreover, melatonin administration reduces the production of VEGF and NO as well as promotes cell proliferation, consequently reducing hypoxia-associated retinopathy [62].

VEGF signaling is a dominant pathway involved in the hypoxia-induced neovascularization and retinopathy in ROP. In the retina, VEGF is primarily secreted by Muller cells and astrocytes. In phase II of ROP, relative hypoxia induces VEGF production and hence promotes pathological vessel proliferation [65, 66]. IGF-1 is another determining factor in the pathogenesis of ROP and is critical in normal retinal vessel growth. IGF-I is a permissive factor of VEGF activation of Akt survival signaling in the growth of endothelial cells in phase I of ROP [67]. In preterm infants after birth, lower levels of IGF-I reduce Akt activation and endothelial cell survival, which makes the maturing avascular retina becomes hypoxic and leads to accumulation of VEGF in the vitreous [67]. IGF-1 level rises with high levels of VEGF, which triggers neovascularization through the activation of MAPK and Akt signaling pathways in phase II of ROP [67, 68]. Inhibition of IGF-1 and VEGF are capable to hinder the neovascularization.

6. Antioxidants in Ischemic Retinopathies

DR and ROP are blinding disorders that affect working-age adults and premature infants, causing enormous burden on the society. Yet, the treatment options are limited to laser photocoagulation and anti-VEGF therapy. Pathologically, they both follow a similar pattern as in ischemic retinopathy. As oxidative stress play a pivotal role in the pathogenesis of ischemic retinopathy, agents that can reduce the formation of ROS or increase the antioxidant mechanisms to counteract oxidative stress are beneficial to the situation. Examples of some of these agents are discussed below.

Catalase and SOD are potent ROS scavengers to block oxidative stress in the retina. SOD catalyzes the conversion of superoxide to oxygen and H_2O_2 while catalase prevents the formation of hydroxyl radical resulting from the reaction of H_2O_2 and ferrous ions [69]. Gene transfer of plasmids encoding MnSOD or catalase complexed with liposomes reduces apoptosis of retinal vascular cell and prevents retinal degeneration induced by retinal I/R [69]. In addition, pretreatment of intravitreal recombinant adenoassociated virus containing the catalase gene (AAV-CAT) efficiently increases the catalase activity and decreases the levels of H_2O_2, 8-OHdG and nitrotyrosine as well as attenuates the I/R-induced retinal function loss, suggesting the beneficial effects of catalase in ischemia/reperfusion-related eye diseases by reducing oxidative stress [70].

As discussed earlier, nitrosative stress also causes detrimental damages during retinal ischemia. NADPH oxidase catalyzes the formation of superoxide from oxygen and NADPH. In ischemic injury in mice, gene deletion of NADPH oxidase reduces the production of ROS, retinal cell loss and glial cell activation, together with an inhibition in the activation of extracellular-signal-regulated kinase (ERK) and NFκB signaling pathways [71]. Moreover, blockade of astrocytic NFκB signaling or administration of NADPH oxidase inhibitors reduces retinal ganglion cell death cocultured with astroglia under hypoxia [72]. Gene deletion of astroglial NF-κB also reduced oxidative stress in retinal I/R in mice [72, 73]. Most importantly, inhibition of NFκB activation by either gene deletion of NADPH oxidase or application of apocynin reduced the retinopathy in ROP and DR animal models [74].

Edaravone is a free radical scavenger which directly scavenges hydroxyl radical and inhibits lipoxygenase activity [75]. It has been shown that application of edaravone can reduce oxidative stress-induced damage by lowering the levels of malondialdehyde (MDA), raising enzymatic activity of SOD, reducing apoptosis of retinal neurons, and preventing retinal functional deterioration in retinal I/R [76]. This suggests that edaravone protects the eye from I/R injury and may carry therapeutic potential in treatment of I/R-induced eye disorders.

Vitamin E is well known to have potent anti-oxidative properties in ocular disease related to retinal I/R, including ROP and diabetic retinopathy [77–81]. It is a lipid peroxyl radical scavenger via breaking the lipid peroxidation chain and acts against oxidative stress [77–81]. In an animal model of retinal I/R injury, reduced levels of oxidative stress marker such as MDA together with an inactivation of caspase-3 but increased levels of GSH were noted with vitamin E treatment [77–81]. In experimental DR models, oral intake of antioxidants, including vitamin E, significantly reduced lipid peroxidation and arrested retinal abnormalities in streptozotocin (STZ)-induced diabetic rats [82]. Intake of micronutrients based on the AREDS formula (vitamin E, ascorbic acid, beta-carotene, zinc, and copper) inhibited the increases in nitrotyrosine and inducible NOS and decreases in MnSOD in STZ-induced diabetic rats [83]. In ROP models, vitamin E can reduce the area of vaso-obliteration [61, 84], preserve retinal function [85] and protect the reduction of outer plexiform thickness [86]. Most importantly, vitamin E supplementation decreased the risk of severe retinopathy and blindness in very low birth weight infants [81]. All this evidence points to a beneficial role of vitamin E in ischemic retinopathy.

Numerous studies have investigated the role of erythropoetin (EPO) in ischemic retinopathy. EPO is a glycoprotein hormone that is involved in the pathogenesis of ROP. EPO level decreases and contributes to vessel obliteration in phase I of ROP; therefore, early administration of exogenous EPO minimizes vessel obliteration and subsequent hypoxia-induced neovascularization and neuron apoptosis by the activation of prosurvival NFκB signaling pathway via the EPO receptor [87]. However, the level of EPO mRNA increases during the neovascularization phase when late EPO application promotes pathologic neovascularization [87]. In addition, increased level of VEGF leads to vaso-obliteration by suppression EPO expression through the Janus kinase/STAT signaling pathway. Application of exogenous EPO inhibits the activation of STAT3 and hence, reduces the area of vaso-obliteration and consequently neovascularization in phase II of ROP [88]. Furthermore, administration of EPO siRNA suppresses neovascularization during phase II of ROP. Therefore, the time of exogenous treatment/inhibition is very critical to the prevention of ROP pathogenesis [89]. Similarly, EPO also exerts its anti-oxidative and protective effects in DR. Low-dose EPO inhibits oxidative stress and nitro-oxidative stress in experimental diabetic retina by reducing Ang-2 expression and pericyte loss as well as restoring pAkt and heat shock protein-27 levels [90]. In addition, EPO therapy decreases the formation of acellular capillaries and the loss of pericytes, which prevents the early vascular changes in DR [90]. EPO treatment also decreases VEGF levels and protects microvascular and neuronal damage in the experimental diabetic retina [91].

Lutein is a member of xanthophyll family of carotenoids and is contained in dark green leafy vegetables, such as spinach and kale [92–94]. It is a powerful anti-oxidant due to its unique chemical structure. It is characterized by having a hydroxyl group attached to each end of the molecule, making it more hydrophilic, and therefore, lutein reacts more strongly with singlet oxygen than other carotenoids [95, 96]. Increasing evidence has shown that lutein is neuroprotective in retinal I/R injury. We show that lutein prevents the increase of nitrotyrosine and PAR and hence, apoptosis and cell loss in inner retinal neuron in an animal model of retinal

I/R [16]. We further show that lutein can directly protect RGC from H_2O_2-induced oxidative stress and cobalt chloride ($CoCl_2$)-induced hypoxia *in vitro* [97]. In retina of diabetic mice, lutein reduces the production of ROS, inactivates the NFκB signaling pathway, and decreases the levels of oxidative markers, thereby preserving the retinal function [98]. Lutein also prevents ERK activation, synaptophysin reduction, BDNF depletion, and consequently neuronal loss in the diabetic retina [99]. However, supplementation of anti-oxidants, including lutein, is ineffective in preventing ROP in preterm infants [100, 101]. Yet, the protective effects of lutein in ROP are still debatable due to the complicated pathogenesis of ROP.

7. Future of Antioxidant Treatment in Ischemic Retinopathy

DR and ROP are two examples of ischemic retinopathy. As mentioned earlier, DR and ROP are blinding disorders that affect working-age adults and premature infants, respectively. The subsequent long-term disability and poor quality of life pose enormous burden on the society. Much research has been conducted, but with no satisfactory outcome. As discussed by Kowluru and Zhong [18], there are serious challenges although promising results are observed in experimental DR models. Firstly, rodents only display the early retinopathy stages but not the later changes seen in patients with diabetic retinopathy. Secondly, DR is a complex diabetic complication; identification of the best molecular pathway for targeted therapeutic studies is not easy. Yet, the administration of the AREDS formula containing various antioxidants to diabetic rats has provided a significant step forward for antioxidant treatment for DR [83].

In ROP, lutein, vitamin E and EPO have all shown hopeful results in experimental models. Similar to the negative results in antioxidant therapy in DR, lutein is not able to yield beneficial effects in two randomized controlled clinical studies [100, 101]. However, vitamin E and EPO have shown some promising outcomes clinically. An early meta-analysis concluded that there is a 52% reduction in the incidence of Stage 3+ ROP with vitamin E prophylaxis in extremely low birth weight (<1000 gm) infants [80]. In a later clinical study, it is concluded that vitamin E is proven to be effective in prophylaxis of ROP development, and vitamin E supplementation relates to lower incidence of ROP [102]. Recently, interventions such as increasing retinal EPO and vitamin E supplements have been suggested [103]. Although well-designed randomized clinical trials are necessary before these interventions can be put into clinical practice, they have provided strong evidence that antioxidant therapy does have a potential role in treatment of ROP.

8. Conclusion

Hypoxia-induced oxidative stress leads to irreversible retinal damage in ocular diseases in which retinal ischemia is a feature. Treatments that can decrease the production of ROS or increase the ROS scavenging ability of the system may be beneficial to the situation. The positive outcomes in administration of AREDS antioxidants in DR experimental models as well as EPO and vitamin E supplements in ROP infants have strongly indicated the potential efficacy of antioxidant in treating ischemic retinopathy with hypoxia-induced oxidative stress.

List of Abbreviations

8-OHdG:	8-hydroxy 2-deoxyguanosine
AGE:	Advanced glycation end-product
AMD:	Age-related macular degeneration
AR:	Aldose reductase
Cu/Zn SOD:	Copper/zinc superoxide dismutase
DR:	Diabetic retinopathy
EPO:	Erythropoietin
GPx:	Glutathione peroxidase
GSH:	Glutathione
H_2O_2:	Hydrogen peroxide
I/R:	Ischemia/reperfusion
JNK:	c-Jun N-terminal kinases
MAPK:	Mitogen-activated protein kinase
MDA:	Malondialdehyde
MnSOD:	Manganese superoxide dismutase
NADPH:	Nicotinamide adenine dinucleotide phosphate oxidase
NFκB:	Nuclear factor-kappa B
NO:	Nitric oxide
NOS:	Nitric oxide synthase
ONOO:	Peroxynitrite
PARP:	Poly(ADP-ribose) polymerase-1
PKC:	Protein kinase C
PTP:	Permeability transition pores
RAGE:	Receptor for advanced glycation end-products
RNS:	Reactive nitrogen species
ROP:	Retinopathy of prematurity
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
VEGF:	Vascular endothelial growth factor.

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Review Article

The Hypoxic Testicle: Physiology and Pathophysiology

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Mammalian spermatogenesis is a complex biological process occurring in the seminiferous tubules in the testis. This process represents a delicate balance between cell proliferation, differentiation, and apoptosis. In most mammals, the testicles are kept in the scrotum 2 to 7°C below body core temperature, and the spermatogenic process proceeds with a blood and oxygen supply that is fairly independent of changes in other vascular beds in the body. Despite this apparently well-controlled local environment, pathologies such as varicocele or testicular torsion and environmental exposure to low oxygen (hypoxia) can result in changes in blood flow, nutrients, and oxygen supply along with an increased local temperature that may induce adverse effects on Leydig cell function and spermatogenesis. These conditions may lead to male subfertility or infertility. Our literature analyses and our own results suggest that conditions such as germ cell apoptosis and DNA damage are common features in hypoxia and varicocele and testicular torsion. Furthermore, oxidative damage seems to be present in these conditions during the initiation stages of germ cell damage and apoptosis. Other mechanisms like membrane-bound metalloproteinases and phospholipase A2 activation could also be part of the pathophysiological consequences of testicular hypoxia.

1. Introduction

Life on earth appeared about 3,000 million years ago when there was practically no oxygen in the atmosphere. It only reached its present level in the atmosphere approximately 350 million years ago (carboniferous period), clearly showing that cellular life on earth was well adapted to hypoxic conditions a long time before the present oxygen-dependent organisms appeared on earth [1]. Thus, anoxic oxidative and reductive chemical processes and their associated regulation were inherent in life before the carboniferous period, helping to explain the evolutionary and molecular basis of present marine and terrestrial animal adaptation to hypoxic conditions [2, 3]. The processes that use oxygen as final electron acceptor in one-electron transference were selected by evolution as a highly efficient mechanism for oxidative processes in cells. These processes, however, inevitably produce reactive oxygen species (ROS) that, in turn, can lead to the formation of reactive nitrogen species (RNS). Both ROS and RNS can modify biomolecules and affect lipids, proteins, and nucleic acids (e.g., [4]).

In multicellular animals, O_2 supply to tissues is generally provided by pressure-driven volume flow in a vascular system. Hypoxia, defined as the condition of low oxygen pressure or content in the environment, organism or tissue, can be the result of atmospheric low oxygen pressure (hypobaric hypoxia), low oxygen content in aquatic environments, or, in the case of organisms or tissues, to a decreased O_2 exchange with the environment or a decreased O_2 supply by the vascular bed.

Most mammals have little tolerance to hypoxia and their response involves the activation of regulatory mechanisms at systemic, tissue, and cellular levels [5]. The mechanisms related to systemic response to hypoxia include an increase in pulmonary ventilation and subsequently a compensatory rise in the capacity of oxygen transport to the different

tissues by increased erythropoiesis [6–8]. At the cellular level, an induction of glycolytic enzymes and glucose transport is produced under hypoxic conditions. This generates an increase in glycolysis that facilitates the production of ATP by this anaerobic metabolic pathway [7, 8]. At the tissue level, a hypoxic condition induces neovascularization or angiogenesis (new blood vessel formation from preexisting vessels) which requires a series of events including proliferation, differentiation, and migration of endothelial cells, vessel formation, and vascular maturation. All this is driven by vascular growth factors (e.g., vascular endothelial growth factor, VEGF) [7–10]. The increase in VEGF secretion and the expression of their receptors in cells exposed to hypoxia is mediated by hypoxia-induced factor 1 (HIF-1) [8, 11]. This is a heterodimeric transcription factor composed of two subunits HIF-1α and HIF-1β that control the expression of numerous proteins related to the cellular hypoxic response [11–14]. The HIF-1β subunit is a constitutive 90 KDa nuclear protein that does not respond to changes in oxygen level, whereas the HIF-1α subunit is a 120 KDa hypoxia-inducible protein [14–17]. Under normoxic conditions, HIF-1α becomes hydroxylated and ubiquitinated and is sent to degradation by the proteasome [15]. HIF-1α prolyl hydroxylases are Fe(II)- and 2-oxoglutarate-dependent dioxygenases which require ascorbate and molecular oxygen for their catalytic activity [13, 14]. In the catalytic center, the ion Fe(II) can be displaced or substituted by other transition metals such as cobalt, nickel, and manganese, with the loss of its catalytic activity. This inhibition of prolyl hydroxylases and the binding to HIF-1α itself generate the stabilization of HIF-1α, mimicking a cellular hypoxic situation [18].

A systemic hypoxic condition can be described in many situations such as high altitude flight [19], diving (human or animal) [20, 21], chronic obstructive pulmonary disease [22], and sleep apnea (e.g., [23]). Because, however, the relation between the above-mentioned conditions and testicular function has received little attention in the literature (see [24] for sleep apnea), in this paper we will address the consequences for the endocrine homeostasis and sperm output in the testis exposed to environmental or local hypoxia (e.g., varicocele or testicular cord torsion). Thus far, features common to these physiopathological conditions are temperature increase and ROS production, two events that could in part explain the changes in cell endocrine function and the decrease of sperm production in men exposed to hypoxia or with a condition that impairs the blood flow to the testis.

2. Environmental Hypoxia and Male Reproduction

With the exception of the unique native population of Himalayas and The Andes, human exposure to high altitude is not a common condition. A deleterious effect on reproductive function has been attributed to exposure to high altitude since the Spanish conquest of America. Some chronicles from the XVI century already stated fertility problems in humans and animals that reached for the first time America's highlands [25]. At present, these fertility changes are observed in mountaineers, workers, and border personnel in situations that imply acute transfer to highlands [26]. However, it seems to be absent in permanent inhabitants of the highlands such as the stable populations of the Andes and Himalaya ranges. These could be at the root of why some of the studies conducted to prove a relationship between highland populations and fertility have often yielded nonconclusive results [26–28]. Most of the studies that have shown alterations of the male component of fertility with hypoxia in humans have described low sperm counts, sperm mobility, and decrease in plasma testosterone after several weeks of exposure [29–31]. In animal models such as rodents, highly vacuolated Sertoli cells, decreased germ cell numbers, pyknotic germ cell, expansion of testicular blood vessels, Leydig cell number reduction, and changes in testosterone levels have been described in hypobaric hypoxia [32–39].

As the hypoxic testis is the focus of our paper, it is worth mentioning that the main testicular functions are related to endocrine secretion and its associated regulation and to the output of functional sperm cells (spermatogenesis). Whereas the endocrine function is mainly accomplished by Leydig cells in the interstitium (Figure 1), the production of mature functional sperms takes place in several structures in the male reproductive system, including the seminiferous tubules (that releases immature spermatozoa) and the epididymis where the spermatozoa mature and are stored. The seminiferous tubules are the place where proliferation of germ stem cells occurs in contact with the basal membrane and basal part of Sertoli cells, progressing and differentiating through meiosis and spermiogenesis in a centripetal direction toward the lumen of the tubules (Figure 1) [60]. Rat seminiferous tubules are thought to be under an O_2 tension lower than the interstitial O_2 tension in normal conditions [12, 61]. In spite of some controversy surrounding the true values of O_2 tension in the seminiferous tubules, it is clear that the testicular interstitial O_2 tension is approximately 20% of the testicular artery blood oxygen pressure (i.e., 12 to 15 mm Hg; [61]). This oxygen tension is expected to decrease under low atmospheric O_2 pressure (hypoxia) or under conditions of reduced blood flow to the testis (e.g., varicocele or testicular torsion). Thus, whereas oxygen distribution in the testis is determined by the testicular microvasculature, the access to oxygen by spermatogenic cells seems to be determined mainly by O_2 diffusion in the interstitium and seminiferous tubules.

2.1. HIF-1α and Oxygen Sensing in the Testicles. When cells are exposed to a hypoxic environment or a transient ischemia is induced in rat testicles, a rapid (within minutes) accumulation of HIF-1α occurs, not accompanied by an increment in HIF-1α mRNA expression [62, 63]. HIF-1α mRNA is expressed in the whole male reproductive tract under physiological oxygen levels [64, 65]. In the testis of rats exposed to hypobaric hypoxia, HIF-1α was prominently expressed in the nucleus of pachytene spermatocytes and to a lesser extent in spermatids and in the lumen of seminiferous tubules

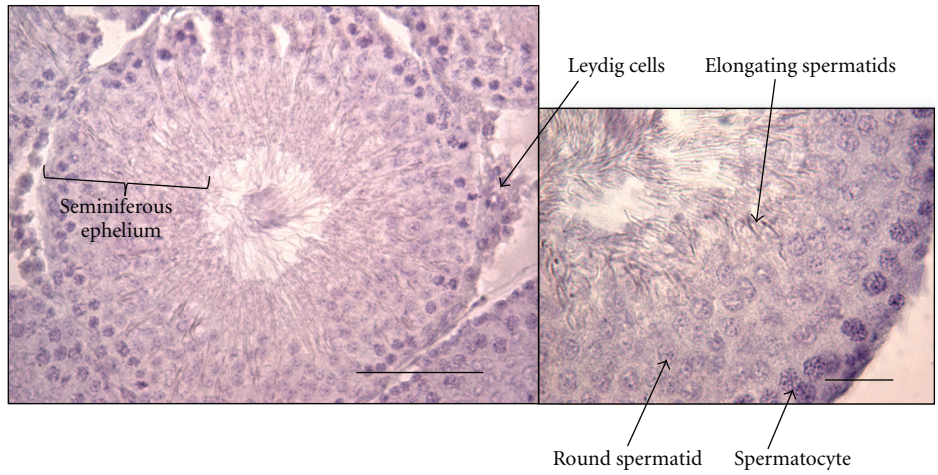

Figure 1: Histological organization of the seminiferous tubules. The figure shows two microscope images of rat testis: (a) low magnification picture of one seminiferous tubule and seminiferous epithelium containing Sertoli and germ cells at different stages of differentiation. Bar 100 μm; (b) a seminiferous tubule section indicating germ cells at different stages of differentiation. Bar 25 μm.

where sperm are located [65]. Out of the vast number of genes induced by HIF-1, one of the most important is VEGF, which has been described in Sertoli and Leydig cells [66]. Its receptor (VEGFR) is present in almost all testicular cell populations, supporting the idea that VEGF could act as a paracrine mitogen and as an angiogenic factor responsible for the modulation of testicular tissue capillarization and testicular capillary permeability [67]. In mice, in vivo application of VEGF to the testis promotes blood capillary formation, but not after the application of antisense oligonucleoties against VEGF [68]. Besides the well-known effects of VEGF as a vascular permeability enhancer and as a mediator of angiogenesis, Hwang et al. observed that VEGF produced an increment in the proliferation of Leydig cells and was an acute inducer of testosterone in a dose-dependent manner [69, 70]. Thus, the molecular infrastructure and the signaling mechanisms for sensing and triggering of a physiological response are present in testicular cells and, as will be shown below, can at least partially explain the physiological and pathological changes associated to environmental and local hypoxia in the testis.

2.2. Early Testicular Vascular Changes Induced by Environmental Hypoxia. In mice, a significant increase in detectable interstitial blood vessels was observed 24 hours after the beginning of normobaric hypoxia [71, 72]. This phenomenon is probably not angiogenic, representing the opening of previously closed shunt vessels. This increase in blood vessels under hypoxia was associated with initiation of vascular cell proliferation. The number of blood vessels detected in the hypoxic testis interstitium continued to increase until day 5 but remained stable after that period [71, 72]. It is likely that the long-term changes in vasculature (>5 days) are associated with angiogenesis, as reported by Farias et al. [34], which is consistent with a sustained raise in VEGF in that period [71, 72].

2.3. Hypoxia and Early Changes in Testicular Steroidogenesis. Gonadotropins released by the hypophysis enter the blood stream to reach the testicle, where Luteinizing hormone (LH) stimulates Leydig cell steroidogenesis in the interstitium, whereas FSH, by stimulation of Sertoli cells, helps to maintain spermatogenesis in the seminiferous tubule. Control mechanisms for FSH secretion seem to be influenced not only by testosterone and its metabolic derivative, estradiol, but also by activins and inhibins produced by Sertoli cells [60].

Little is known about the relationship between hypoxia and steroidogenesis, and the scarce studies performed in humans have been carried out with reduced sample sizes. It has been observed that in a small group of men exposed to an altitude of 4,300 m above sea level, their plasma testosterone level rose by 30% after the third day of exposition [31]. In another study in which 10 mountaineers stayed in the Himalayas for a period of 60 days at 5000 m above sea level, hormonal measures indicated reduced testosterone levels at the end of the period [73].

In experimental mice exposed to normobaric hypoxia, testosterone levels (plasma and intratesticular) were highest at 24 hours for plasma testosterone and 48 hours for intratesticular testosterone. The early increment of both intratesticular and plasma testosterone might be mediated by VEGF, as postulated by Hwang et al. [69] and consistent with a raise in VEGF in mice after 24 hrs of hypoxia [71, 72]. Plasma and testicular testosterone return to normal levels after 48 and 72 hours, respectively [71, 72].

These results are in agreement with published data on early testosterone increments in mountaineers exposed to high altitude or in newborns exposed to neonatal hypoxia [31, 74, 75]. Testosterone has a well-known relaxing effect on smooth muscle which can induce a vasodilator effect in minutes [76–78], an effect that, hypothetically, could be part of the hypoxia response mechanisms in the testicles

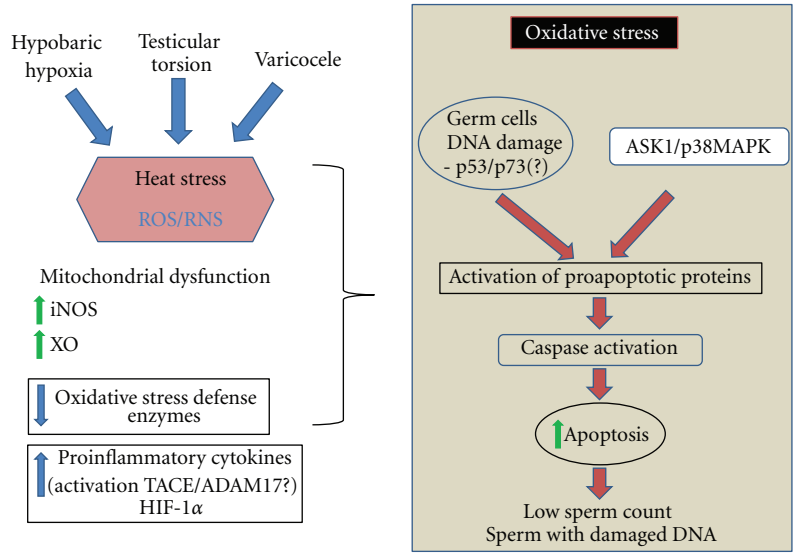

Figure 2: Diagram of molecular and cellular events triggered by hypobaric hypoxia (HH), testicular torsion (TT), and varicocele (Var). This model suggests that HH, TT, and Var have a common mechanism of action at the testicular level by inducing oxidative stress owing to an increase in reactive oxygen and nitrogen species (ROS/RNS) formation and impairment in the oxidative defense mechanisms. Experimental evidence points to heat stress in HH and Var, but this parameter has not been determined in TT. The increase in ROS/RNS is probably owed to mitochondrial dysfunction along with activation of enzymes such as xanthine oxidase (XO) or the inducible nitric oxide synthetase (iNOS). Oxidative stress induces activation of p53, p73, and ASK/p38 MAPK, which stimulate the activation of proapoptotic proteins (e.g., BAX) that in turn will lead to caspase activation and increase in germ cell apoptosis. The induction of proinflammatory cytokines is probably part of the response mechanism to cellular damage.

[71]. Testosterone seems to have a relevant role in high altitude adaptation owing to its identity as an erythropoietic hormone which acts directly on bone marrow at the level of polychromatophilic erythroblasts [79]. Thus, testosterone administration has been shown to stimulate the production of red blood cells in males, especially elderly males, and it is associated with the increment of hemoglobin that occurs during puberty in young men [80, 81].

Thus, an early rise in testosterone in hypoxia and its role as a vasodilation agent is consistent with its possible role in early vascular changes in the hypoxic testis, as well as its being a likely coactivator of the erythropoietic response in hypoxia, acting both as a local paracrine hormone and as an endocrine signal toward bone marrow cells.

2.4. Chronic Intermittent Hypobaric Hypoxia: Testicular Histological and Endocrine Changes. Hypobaric hypoxia is a stress factor that generates a series of physiological changes in order to compensate for environmental low partial oxygen pressure. Exposure to low levels of environmental oxygen and high altitude tend to trigger chronic mountain sickness (CMS) in humans and animals not genetically adapted (GA) to high altitude, for example, llamas or alpacas. The absence of CMS in GA or nonGA animals or humans appears to be linked to various adaptations involving certain patterns of gene expression [82, 83].

With regard to the effects of environmental hypoxia on male infertility, chronic hypoxia induces a state of reversible oligozoospermia in healthy men [30]. Previous studies on nonGA male rats indicated that chronic intermittent hypoxia reduces sperm motility and the sperm count in semen [41, 84]. A reduced sperm count can be related to the increase in germ cell apoptosis promoted by this hypoxic condition [34, 39]. The same results were observed in male rhesus monkeys [85]. Morphological studies have revealed that chronic hypoxia causes degeneration of the germinal epithelium, folding of the basement membrane, degeneration and detachment of germ cells, changes in lipid droplets in Sertoli cells, and an increase in lipoperoxidation [34, 39]. Other local changes in the testicles have also been observed, including an increase in vascularization, an increase in testicular temperature, a decrease in testicular mass, and an increase in interstitial space [34, 71].

The mechanism by which permanent decreases in oxygen supply generate impairment in germ cell development and death is probably mediated by an increase in intratesticular or seminal ROS (Figure 2). Although these molecules have a physiological role in the spermatogenic process, a pathological increase in their numbers would negatively affect the survival and differentiation of germ cells [86].

On the endocrine side, plasma and testicular testosterone in mice are significantly diminished at 20 days of normobaric hypoxia compared with day zero [71], similarly to what has been described in hypobaric hypoxic animals [31, 37]. This effect of testosterone reduction associated with long and intermittent periods of hypoxia has been observed even in patients suffering from obstructive sleep apnea [87] and in long-term exposure to high altitude in humans [73] and is consistent with changes in the Leydig cell population in experimental animals subjected to this condition [32, 34, 71].

Table 1: Experimental approaches that prevent testicular and sperm damage during environmental hypoxia.

Hypobaric hypoxia	Treatment	Mechanism	Results versus injury	Reference
Acute	Cyproheptadine	5-HT receptor blocker	▼Effects on spermatogenesis and testosterone production	[40]
Chronic	Caloric restriction	?	Suppresses early rise in testosterone	[31]
Intermittent	Ascorbic acid	▼Oxidative stress in testis and epididymis ▲Glutathione reductase activity in testis and epididymis ▲ Sperm count	Reversed the effects of oxidative stress in testis, epididymis, and sperm cells	[41]
Intermittent	Melatonin (oral administration)	Prevents oxidative damage of enzymes like superoxide dismutase, catalase, and glutathione peroxidase	Protective effect against lipid peroxidation under oxidative stress and reduction in sperm motility	[42]
Intermittent	Melatonin (intraperitoneal administration)	▼ Levels of LH and FSH Inhibition of aromatase	No protective effect in testis, and epididymis No prevention of reduction in the numbers of sperm cells	[43]
Chronic and intermittent	Ibuprofen	Decreases hypoxia-induced vasodilation?	Protective effect against hypoxia-induced seminal lipid peroxidation	[42]

2.5. The Temperature-ROS Connection and Spermatogenic Cell Damage in Environmentally Hypoxic Testicles. ROS production requires O_2 as a substrate. Counter intuitively as it seems, hypoxia, as several studies have reported, can increase total intracellular ROS production in cells and tissues [88–93]. Consistently with this, evidence of ROS modification of proteins and nucleic acids has also been reported in yeast exposed to hypoxic conditions [94]. Environmental hypoxia leads to vascular changes that are associated with an increase in testicular temperature (1.5°C on average, [35]). This condition was linked to oxidative stress and was prevented by antioxidant treatment [41, 42]. Although the pathological role of oxidative stress in male reproduction induced by environmental hypoxia seems well established (see also Table 1), the associated rise in temperature needs to be considered for an understanding of the consequences of hypoxia on testicular function. As mentioned before, the changes in subscrotal temperature were relatively mild in hypobaric hypoxia (1.5°C on average) [35]. If, however, this condition is to be maintained chronically during the hypoxic period it becomes similar to temperature increases in pathological conditions like varicocele (see below). Thus, in the following paragraphs, we will review experimental testicular hyperthermia albeit that the protocols used have gone from mild temperature changes (35°C) for 24 hrs to acute (30 min) severe hyperthermia (43°C). It is worth noting that the experimental interventions mentioned earlier consist in external temperatures applied to the scrotum. The true intratesticular temperature was in most cases unknown (see [95] for a review).

Heat stress induces general changes in the transcriptome of mice and human testes, and a total of 67 transcripts were found to be heat regulated in C57BL/6 mice [96]. Another study in mice with a heat stress protocol of 35°C for 24 h showed that 225 genes were differentially expressed between fertility-related heat-susceptible and heat-resistant animals [97]. On the other hand, in humans, it was shown that 31 and 36 known proteins were differentially expressed two and nine weeks after heat treatment, respectively [98]. Although the range of functions that the genes upregulated or downregulated by heat is broad, many of these genes are associated with heat stress, cell signaling, and apoptosis.

Under normal conditions, the highest rate of germ cell apoptosis is observed in early zygotene and ending pachytene spermatocytes (stages I and XII for rats and mice). The evaluation of germ cell apoptosis one to two days after heat stress showed a significant increase in apoptosis, mainly in the early (I–IV) and late (XII–XIV) stages. Pachytene spermatocytes, dividing spermatocytes, and early spermatids were the most frequent cell types observed undergoing apoptosis [99]. Eventually, spermatogenesis recovers to levels similar to those in nontreated animals because spermatogonia are relatively heat resistant, with the exception of B-type spermatogonia of rams [99, 100] and bulls [101]. Interestingly, isolated haploid germ cells but not somatic cells undergo apoptosis at 37°C under the same culture conditions, strongly suggesting that heat stress activates the apoptotic pathway mainly in germ cells [102].

The mechanism by which heat stress induces apoptosis in germ cells has yet to be defined. In rats and monkeys heat stress induces translocation of the proapoptotic protein BAX from the cytoplasm to the mitochondria (Figure 2), where it helps to release cytochrome c [103–107]. During heat stress, caspase-9 and 3 (hallmarks of apoptosis) become active, and their pharmacological inhibition prevents germ cell death, suggesting that caspases are directly linked to germ cell death after heat stress [104, 105, 108].

In the testis, the generation of ROS seems to be of paramount importance in germ cell apoptosis and DNA damage [109]. At physiological levels, ROS are essential for normal

reproductive functioning, acting as metabolic intermediates and regulating vascular tone, gene expression, and sperm capacitation [109, 110]. Heat stress induces oxidative stress, triggering cell survival or apoptosis depending on the cell type and the extent of the insult (Figure 2). This heat stress appears related to ROS-generating enzymes that produce ROS as by-products of their enzymatic activity. Xanthine oxidase (XO) catalyzes the conversion of hypoxanthine and xanthine to uric acid, producing hydrogen peroxide as a by-product, and XO inhibitors suppress testicular germ cell apoptosis induced by experimental cryptorchidism (testis subjected to the core body temperature) [111]. There is a lack of information, however, about whether or not other ROS-generating enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), NADPH oxidase (NOX), and the mitochondrial NADH-CoQ oxido-reductase are activated after testicular heat stress. In other oxidative processes, nitric oxide (NO) is synthesized intracellularly through the action of a family of nitric oxide synthetase (NOS) enzymes. These NOS enzymes catalyze the NADPH- and O_2-dependent oxidation of L-arginine to L-citrulline, producing NO [112]. This molecule is a free radical and is chemically more stable and less reactive than other ROS such as the superoxide anion or hydrogen peroxide [113]. Furthermore, NO in the presence of ROS can form the highly reactive oxidant peroxynitrite [114]. In monkey testes, endothelial nitric oxide synthetase (eNOS) and inducible NOS (iNOS) were found to be expressed in Sertoli and germ cells. No obvious alterations in eNOS levels were detected after heat stress, but the levels of iNOS increased three days after heat treatment compared with the controls showing a robust increase in iNOS expression in germ cells [115]. Thus, heat stress seems to induce NO production and it might contribute to oxidative damage in germ cells (Figure 2). The molecular targets that are modified by NO production and the consequences of this RNS in testis physiology and pathophysiology are still unknown, however.

As described above, many studies have been performed on experimental hyperthermia in animals, showing the triggering of apoptosis in pachytene spermatocytes [116, 117]. In contrast, few studies of hyperthermia and its associated physiological and biochemical changes have been performed in isolated cells. The only study found in the literature using prepubertal monkey Sertoli cells [118] reported that adherent junction-associated proteins are downregulated by high temperatures (43°C). Conversely, vimentin expression is upregulated by high temperatures. These changes in cytoskeletal and junction proteins were thought to be associated with a marked decrease in androgen receptor (AR) expression after heat treatment. As was clearly demonstrated using selective Sertoli cell AR KO mice, testosterone is essential for spermatogenesis through its actions on Sertoli cells [119, 120]. Thus, Sertoli cell AR downregulation by hyperthermia explains, at least in part, spermatogenic arrest in testis subjected to high temperatures (and hypoxia?). In spite of the mentioned correlation of AR expression and hyperthermia, evidence showing the mechanisms connecting AR downregulation and spermatogenic cell apoptosis under conditions of high temperature is lacking. In relation to testosterone, studies of the effects of high temperature on Leydig cell function and testosterone production are apparently absent in the literature. With regard to isolated germ cells, [121] showed that increasing the incubation temperature of rat pachytene spermatocytes and round spermatids to 37 to 40°C increased intracellular [Ca^{2+}] ([Ca^{2+}]i) to levels that modify signaling in these cells [122]. The intracellular pH in these cells was found to decrease with increasing temperature. These changes occurred within one minute of the increase in temperature and can be classified as early events in the response of these cells to heat stress. Interestingly, the same combination of changes in these cellular parameters (increase in [Ca^{2+}]i and decrease in intracellular pH) was associated with apoptotic cells in the testis [123], strongly suggesting that high temperatures per se can set physiological conditions in spermatogenic cells that make them prone to other noxious or proapoptotic stimuli from Sertoli cells. Furthermore, we recently found that a high temperature (40°C) induced a rapid increase in reactive oxygen and/or nitrogen species in pachytene spermatocytes but not in round spermatids (Pino, Osses, Oyarzun, Farias, Moreno and Reyes, unpublished results), providing the possible noxious stimuli that could differentially trigger cell death in spermatocytes. Thus, oxidative stress seems to be at the root of the cell changes in the environmental hypoxic testicle that lead to spermatogenic cell death. This stress seems to be compounded by the rise in testicular temperature, especially in chronic hypoxia.

Hypoxia, by triggering in most cells and tissues an HIF-1α-dependent response, can induce metabolic adaptations but, on the other hand, hypoxia starts a relatively complex spectrum of responses that involves not only HIF-controlled signals and gene expression but also ROS/RNS, AMPK, and PLA2 activation that appear to determine tissue-specific effects of the hypoxic state [124]. In relation these responses, it has been known for almost 20 years that PLA2 and AA seem to play a role in tissue injury and response under hypoxia-reoxygenation situations in animal tissues [125–127]. In the testis, Sertoli cells (SCs) produce arachidonic acid (AA) and some of its metabolites in an FSH-regulated manner, [128]. In a SC-derived cell line (TM4), activation of CD95 (Fas), a pathway known to participate in spermatogenic cell apoptosis [123, 129], can activate cytosolic PLA_2 and AA release [130]. Our unpublished results (Madrid, Osses, Pino, Oresti, Paillamanque, Moreno and Reyes) show that AA can induce apoptosis in spermatogenic cells, together with increases in intracellular Ca^{2+} and a lowering of intracellular pH, two proapoptotic conditions in these cells [123]. Thus, although still not tested experimentally, hypoxia-induced PLA_2 activation in Sertoli cells and AA release are possible mechanisms by which hypoxia (environmental and local) could also help to trigger spermatogenic cell death in the hypoxic testis.

Some of the cellular and molecular mechanisms of environmental hypoxia-induced sperm and endocrine changes discussed above are apparently corroborated by some treatments that can partially reverse the effects of hypoxia (Tables 1, 2, and 3). Thus, in environmental hypoxia, a serotonin (5-HT) blocker can reverse the effects of hypoxia on spermatogenesis and testosterone production, in agreement with

Table 2: Pharmacological approaches that modify testicular and sperm functional parameters in varicocele.

Treatment	Mechanism	Result versus injury	Reference
Polydeoxyribonucleotides	Adenosine A2A-receptor stimulator	▼ Histological changes produced by varicocele ▲ Microvessels	[44]
Aminoguanidine	Nitric oxide synthase inhibitor	Prevents sperm DNA fragmentation	[45]
Aminoguanidine	Nitric oxide synthase inhibitor	▲Sperm vitality, motility, and morphology	[45]
EGF	Growth factor	▲ Sperm count and motility	[46]
Melatonin	Antioxidant, hormone	▼ MDA and Bax levels ▲ CAT, SOD, and GPx activities	[47]

CAT: catalase; GPx: glutathione peroxidase; GSH: glutathione; SOD: superoxide dismutase.

Table 3: Treatments that improve and/or prevent testicular and sperm damage in experimental testicular torsion.

Treatment	Mechanism	Results versus injury	Reference
Transplanted endotelial progenitor cells	N/A	▼ Apoptosis Prevents histopathological damage	[48]
Pretreatment w/ginkgo biloba (EGb 761)	Extract of dried leaves	Prevents histopathological damage ▼ Apoptosis, eNOS mark ▲ Mean seminiferous tubule diameter	[49]
Pretreatment w/sildenafil citrate (0.7 mg/kg)	Phosphodiesterase type 5 inhibitor	▼ MDA levels and eosinophil counts Prevents histopathological damage ▲ GSH, PON1, NO, and blood lymphocyte counts in plasma	[50]
Pretreatment w/sildenafil citrate (1.4 mg/kg)	Phosphodiesterase type 5 inhibitor	▼ MDA levels ▲ PON1, vitamin E, β-carotene in plasma, and GSH levels	[50]
Pretreatment w/melanocortin 4 activator	Melanocortin analog	▼ IL-6 and TNF-α, Bax ▲ Bcl-2 expression, Johnsen's spermatogenesis score	[51]
Rutin	Antioxidant	▼ MDA levels ▲ SOD and CAT activities, Johnsen's spermatogenesis score	[52]
Gradual detorsion	N/A	▲ SOD and GPx activities	[53]
Molsidomine	Nitric oxide donor	▼ MDA levels and Cosentino's score ▲ Sonic hedgehog and HIF1-α expression	[54]
Cyclosporine and FK-506	Immunophilin ligands	▼ MDA levels, apoptosis ▲ CAT, SOD, and Gpx activities	[55]
Pretreatment w/trapidil	Vasodilator	Prevent histopathological damage	[56]
Hemin	Iron-containing porphyrin	▼ NF- kappaB and ERK levels ▲ heme oxygenase-1	[57]
Pretreatment w/ethyl pyruvate	Antioxidant, anti-inflammatory	▼ MDA, myeloperoxidase levels and apoptotic index ▲ CAT, GSH, Gpx, SOD activities and sperm count and motility	[58]
Pretreatment w/losartan, lisinopril	Angiotensin II receptor antagonist, ACE inhibitor	Prevents histopathological damage in contralateral testis	[59]

ROS: reactive oxygen species; GSH: glutathione; SOD: superoxide dismutase; eNOS: endothelial nitric oxide synthetase; MDA: malondialdehyde; CHOP: C/EBP homology protein; CREMτ: cAMP-responsive element modulator-τ; MCP: monocyte chemotactic protein-1; N/A: not available.

the effects of 5-HT on testicular vasculature and testosterone production [40]. The effect of ibuprofen that appears to decrease oxidative stress has been attributed to its anti-vasodilatation actions in the testicle [42]. Furthermore, antioxidant treatment can also partially reverse the effects of hypoxia in testis epididymis and sperm [41]. The apparently contradictory results in terms of hypoxic changes in the testis obtained by using melatonin in different routes of administration [41, 42] is puzzling, but this compound has both antioxidant and hormonal properties. How

the administration route systemically affects these two properties of melatonin is unknown.

3. Local Hypoxia: Varicocele and Testicular Torsion

3.1. The Hypoxia-ROS-Temperature Connection in Varicocele. An important pathology associated with male subfertility and an increase in intratesticular and seminal ROS levels is varicocele, which is characterized by abnormally dilated veins in the pampiniform plexus, and it has been associated with abnormalities in semen analyses. Varicocele is diagnosed only in humans and it seems to be associated with the erect position whereby one-way valves in the spermatic veins insure the exit of waste products against gravity [131]. Dysfunction of the internal spermatic vein valves with age increases the pressure up to eight times above the physiologic level in the venous drainage, which deviates testicular venous flow to other horizontal routes. This unique biological phenomenon causes hypoxia and oxidative stress, which severely impairs spermatogenesis [132]. A diagnosis of varicocele is made in 35% of men with primary infertility and in approximately 80% of men with secondary infertility [133, 134]. Varicocele can cause tissue hypoxia and related events such as angiogenesis by promoting expression of HIF-1α, which upregulates VEGF and allows expression of different adaptive cellular mechanisms in response to hypoxia [135–137]. HIF-1α has been detected in the cytoplasm of germ cells and vascular endothelium [137]. Furthermore, increased VEGF expression has been observed in testicular endothelial cells in men with varicocele and in the germ cell cytoplasm in rats with experimental varicocele (Figure 2). This elevated expression of VEGF is likely to have a paracrine effect on the testicular microvasculature, inducing the growth of new vessels, as has been observed in men with varicocele [134]. Thus, upregulation of HIF1α seems a feature common to varicocele, hypobaric hypoxia, and experimental heat stress alike (Figure 2).

An alternative hypothesis, however, is that high temperature rather than intratesticular hypoxia is the main source of oxidative stress in patients with varicocele, which is supported by three lines of evidence: (1) patients with varicocele have a higher scrotal temperature and shorter recovery period after cold stress [138]; (2) varicocelectomy improves semen parameters, probably by reducing testicular temperature and ROS levels and increasing antioxidant activity in the seminal plasma [139–141]; (3) increased scrotal temperature but not varicocele grade correlates well with testicular oxidative stress and apoptosis [142].

It is generally accepted that testicular and seminal ROS levels are important in terms of the deleterious effects of varicocele on male fertility [109, 143–145]. In fact, H_2O_2 applied directly in the testis induces germ cell apoptosis, showing that oxidative stress may directly induce apoptosis in germ cells [146]. ROS levels in semen samples from men with varicocele are significantly higher than those of fertile control men [143, 144, 147], and they are directly related to the degree of varicocele [148]. In this regard, the damage caused by varicocele becomes worse as the time between its first appearance and varicocelectomy increases [149], indicating that the testes have intrinsic mechanisms for avoiding permanent damage under certain conditions, but that damage is inevitable when the hypoxia (or elevated temperature) is sustained over time. Changes in testicular tissue have been described in both humans and animal models of varicocele, particularly in rats, producing smaller testicles with a decrease in Leydig cell functioning and a low total sperm count but with no abnormalities in the motility or morphology of the spermatozoa [150–152].

Different intracellular pathways are activated downstream of ROS production, among which the ASK1/p38-MAPK pathway has been shown to be important in germ cell apoptosis after heat stress [153–155]. p53, a master gene in apoptosis and a target of p38 MAPK (Figure 2), is activated in rats with experimental varicocele [156]. In fact, the proapoptotic gene Bax, which is a transcriptional target of p53, is upregulated in experimental varicocele in animal models [47, 157–159]. In addition, the downregulation of Bcl-2 and increased expression of caspase-9 and activated caspase-3 in the ipsilateral testis at eight and 12 weeks after the onset of varicocele has been documented, indicating gradually increased testicular tissue apoptosis through the intrinsic pathway [158]. Therefore, elevated oxidative stress may lead to an elevated rate of germ cell apoptosis, which could explain the decrease in sperm count reported in infertile patients with varicocele.

3.2. Testicular Torsion: Hypoxia-ROS and Inflammation. Testicular torsion is a urological emergency condition causing pain and eventually leading to total loss of the testis [160, 161]. It consists in the twist or rotation of the vascular pedicle, and the damage to the testis depends on the degree of ischemia/reperfusion. It occurs annually in 1/4,000 males younger than 25, affecting 1/160 males by the age of 25 [160, 161]. Animal model studies have shown that 720 degree torsion induces ischemia sufficient to disrupt the seminiferous epithelium [162–164]. Testicular salvage depends on the degree of torsion and on the time that has elapsed until repair. Detorsion within 6, 12, and 24 hours of torsion results in a salvage rate of 90%, 50%, and less than 10%, respectively [165]. Experimental testicular ischemia/reperfusion (IR) (e.g., torsion/detorsion) in rats and/or mice induced a decrease in germ cells, vacuolization of the seminiferous epithelium, decreases in sperm production, and germ cell apoptosis [162–167], a pattern of effects very similar to those of chronic environmental hypoxia, as discussed above. Several studies have pointed out the importance of ROS production in the onset of testicular IR response, as evaluated by thiobarbituric acid reactive substances (TBARSs) or 8-isoprostane levels [146, 168, 169]. Interestingly, IR promotes the recruitment of neutrophils to subtunical venules in the testis. Since neutrophils are a source of ROS in many other conditions, it is possible that their presence contributes to oxidative stress, which in turn contributes to germ cell DNA damage and germ cell demise by either apoptosis or necrosis [146, 168, 170]. IR induces several bona fide

apoptosis markers such as DNA fragmentation, activation of caspase-3, caspase-9, and release of cytochrome C from mitochondria and upregulation of BAX [166, 171, 172]. Although general caspase inhibitor and caspase-9 inhibitor prevent germ cell apoptosis in IR testis, it seems that necrosis may also contribute to germ cell demise [173]. In fact, germ cells can trigger both the necrotic and the apoptotic program depending on the stimuli and the time that has elapsed since the injury [170]. Thus it is possible that germ cell necrosis may be an early stimulus in order to recruit neutrophils that eventually promote oxidative stress and the induction of apoptosis.

IR produces an increase in the proinflammatory cytokines TNFα and IL-1β (Figure 2), which suggests a role for these cytokines as early mediators of injury in the testis [169]. Upregulation of TNFα and IL-1β is detected as early as 0.5 h after IR in mice testes, and this precedes the activation of c-jun N-terminal kinase (JNK) along with two downstream transcription factors; ATF-2 and c-jun in intratesticular blood vessels. E-selectin is a transcriptional target of ATF-2 and c-jun, which could explain its upregulation in IR testes [146, 168, 169]. These results suggest that an increase in TNFα and/or IL-1β after IR of the testis stimulates the activation of the JNK signaling pathway leading to the expression of E selectin in endothelial cells and ultimately neutrophil recruitment. Interestingly, TNFα and/or IL-1β can recruit neutrophils in parenchymal testis veins, suggesting the crucial role of these cytokines in the response of testes after IR insult [169, 174]. TNFα is expressed as a transmembrane protein in pachytene spermatocytes, round spermatids, and testicular macrophages [175]. Consequently, the release of TNFα from the cell surface (shedding of the extracellular domain) seems to be an important step in the molecular cascade activated in IR.

The family of membrane-bound metallo proteinases known as metalloproteases and disintegrins (ADAMs) has a central role in juxta/paracrine and autocrine signaling by controlling the ectodomain shedding of different ligands and receptors such as epidermal growth factor (EGF) or TNFα [176]. We have found that ADAM17, the main sheddase of TNFα, is expressed in germ cells, and its activity is necessary to induce apoptosis in physiological conditions [177]. In addition, ADAM17 and ADAM10 are upregulated after genotoxic damage and their pharmacological inhibition prevents germ cell apoptosis [178, 179]. What is more, significantly higher levels of TNFα have been found in semen samples from infertile patients as compared with controls, suggesting a role for this cytokine in male fertility in general and specifically in IR-induced pathophysiological changes in the testis [180–182]. Therefore, it is not far-fetched to propose hypothetically that IR associated with testicular torsion could induce activation of ADAM17 and shedding of TNFα as a primary response to hypoxia and oxidative stress.

A range of pharmacological agents can diminish the deleterious effects of varicocele and testicular torsion on several testicular and semen parameters (Tables 2 and 3). In spite of the pharmacological diversity observed in those studies, however, a certain pattern does emerge from the studies cited in Tables 1, 2, and 3. First, antioxidants improve testicular function in environmental and local hypoxia, in agreement with the proposed role of ROS in testicular pathogenesis in these conditions. Second, the possible protective role of the cAMP signaling pathway is strongly suggested by the studies performed on varicocele and testicular torsion. In organ models of IR (liver, heart) the cAMP signaling pathway has been shown to be protective in IR-induced cell apoptosis [183, 184]. Interestingly, no such approach has been tested in environmental hypoxia studies. Third, a possible role of NO is suggested by the studies in varicocele and testicular torsion, although the results in these pathologies are contradictory. Again, no studies on the role of NO in testicular effects have been performed on environmental hypoxia.

4. Concluding Remarks

The available data suggest that oxidative and heat stresses are common features of hypobaric hypoxia, varicocele, and testicular torsion. These conditions induce also the activation of the antioxidant defence mechanisms that once overflown promotes activation of apoptosis and DNA damages in the developing germ cells. It is unknown, however, whether heat stress is the cause or consequence of these pathological conditions. Experimental evidence clearly shows that experimental heat stress mimics some of the pathways and cellular responses observed in hypobaric hypoxia, varicocele, and testicular torsion, but there is still a lack of direct evidence about the real contribution of this parameter to these pathological conditions.

Some of the cellular and molecular mechanisms of environmental hypoxia-induced sperm and endocrine changes discussed above seem to be corroborated by some treatments that can partially reverse the effects of hypoxia (Tables 1, 2, and 3). New experimental evidence suggests, however, that previously overlooked molecules such as AMPK, PLA2, arachidonic acid, and ADAM17 may be important players in the onset of testicular damage in the hypoxic testis, and therefore they could constitute new pharmacological targets in the design of experimental strategies to prevent germ cell damage and decrease fertility under these conditions.

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Research Article

PGC-1α Induction in Pulmonary Arterial Hypertension

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Idiopathic Pulmonary arterial hypertension (IPAH) is characterized by the obstructive remodelling of pulmonary arteries, and a progressive elevation in pulmonary arterial pressure (PAP) with subsequent right-sided heart failure and dead. Hypoxia induces the expression of peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) which regulates oxidative metabolism and mitochondrial biogenesis. We have analysed the expression of PGC-1α, cytochrome C (CYTC), superoxide dismutase (SOD), the total antioxidant status (TAS) and the activity of glutathione peroxidase (GPX) in blood samples of IPAH patients. Expression of PGC-1α was detected in IPAH patients but not in healthy volunteers. The mRNA levels of SOD were lower in IPAH patients compared to controls (3.93 ± 0.89 fold change). TAS and GPX activity were lower too in patients compared to healthy donors, (0.13 ± 0.027 versus 0.484 ± 0.048 mM and 56.034 ± 10.37 versus 165.46 ± 11.38 nmol/min/mL, resp.). We found a negative correlation between expression levels of PGC-1α and age, PAP and PVR, as well as a positive correlation with CI, PaO_2, mRNA levels of CYTC and SOD, TAS and GPX activity. These results taken together are indicative of the possible role of PGC-1α as a potential biomarker of the progression of IPAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a complex disorder characterized by the obstructive remodelling of pulmonary arteries, leading to a progressive elevation of pulmonary arterial pressure (PAP) and subsequent right-sided heart failure and death [1]. There are five categories in which pulmonary hypertension (PH) diseases can be grouped according to specific therapeutic interventions directed at dealing with the cause of (1) PAH, (2) pulmonary hypertension with left heart disease, (3) PH associated with disorders of the respiratory system or hypoxemia, (4) PH caused by thrombotic or embolic diseases, and (5) PH caused by multifactorial mechanisms [2]. Idiopathic PAH (IPAH) is included in group 1 and within patients with a mean pulmonary artery pressure (PAPm) ≥ 25 mmHg, and a pulmonary capillary wedge pressure (PCWP), left atrial pressure, or left ventricular end-diastolic pressure ≤ 15 mmHg, and a pulmonary vascular resistance greater than three Wood units [3]. Remodelling of pulmonary arteries leads to an increase of pulmonary vascular resistance (PVR) which produces right ventricular (RV) overload, hypertrophy and dilatation, and eventually RV failure and death [4]. These changes are due to an inadequate adaptation of myocardial contractility [5].

Although physiopathology of IPAH remains under investigation, the role of radical oxygen-mediated events, including myocardial ischemia, seems clear [6]. During the progression of PAH, there is a progressive hypoxia situation originated as a consequence of an increase in the demand of oxygen by hypertrophied cardiomyocytes, as well as a reduction in the capillary density [7, 8]. This hypoxia situation leads to an imbalance in oxidative/antioxidative status with

subsequent cellular damage which contributes to RV failure [9, 10].

The increase in the production of the reactive oxygen species has been established in different experimental animal models of PH and in IPAH-diagnosed patients [11, 12]. The main source of these species, in particular $O_2^{\bullet -}$, is the injured vasculature which results in impaired nitric oxide (NO) signaling and the development of pulmonary vascular remodeling [13, 14]. In this context, the role of superoxide dismutase (SOD) is relevant, because it is involved in the regulation of NO metabolism and in preventing PH, as it has been described in adult animal models [15]. Another important antioxidant enzyme involved in oxidative enzymopathies (including PH) is the glutathione peroxidase (GPX). A deficit in this enzyme is associated with an increase of reactive oxygen species and a decrease of $NO^{\bullet}$ which leads to endothelial dysfunction and impaired vascular reactivity [16].

Peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α) is a well-known regulator of the transcription of genes involved in oxidative metabolism and mitochondrial biogenesis, including the mitochondrial respiratory chain CYTC [17]. This transcriptional coactivator plays a key role in the metabolic control of the cardiac muscle and participates in cardiomyocyte differentiation [18]. PPAR agonists (pioglitazone and rosiglitazone) preserve both ventricular function and PGC-1α levels [19–21]. The tissue's capacity to produce PGC-1α after an hypoxic event, could predict the regenerative capacity of the tissue. In fact, we have recently reported that expression levels of PGC-1α in blood samples of patients with myocardial infarction can be correlated with the size of the hypoxic area, supporting the role of this protein in protecting myocardiocytes after hypoxia injury [22].

The main objective of this study is to analyze the expression levels of PGC-1α in 12 IPAH-diagnosed patients and in 15 healthy volunteers. These levels are correlated with the progression of the disease, with cytochrome c (CYTC) and superoxide dismutase (SOD) mRNA levels and with total antioxidant status (TAS) and glutathione peroxidase (GPX) activity.

2. Materials and Methods

2.1. Patients. In this study 12 IPAH-diagnosed patients were compared with 15 healthy volunteers. Inclusion criteria for the 12 diagnosed patients included an mPAP > 25 mmHg, a PWP less or equal to 15 mmHg and a PVR > 3 Wood units measured by catheterization. Clinical features of patients included in this study are summarized in Table 1. All patients received different combinations of bosentan, treprostinil, nifedipine, and iloprost before sample collection. Healthy volunteers were paired in age with patients (51.34 ± 8.28 and 56.5 ± 3.23 years old, resp.).

All experiments were approved by the local ethics committee and informed consent was obtained. On the one hand 2.5 mL of peripheral blood were collected in PAX gene RNA collection tubes (Qiagen, Valencia, CA, USA) and stored at −80°C until its analysis, as recommended by the manufacturer. On the other hand, 4 mL of peripheral blood was collected in EDTA vacutainers (Becton Dickinson, NJ, USA). Plasma was isolated by centrifugation (15 minutes at 2500 rpm) and stored at −80°C in 500 μL aliquots.

2.2. Determination of PGC-1α, CYTC, and SOD mRNA Expression. Total RNA was extracted from peripheral blood stored in PAX gene collection tubes using the PAXgene blood RNA kit (Quiagen, Valencia, CA, USA), according to manufacturer instructions. RNA concentration was determined by spectrophotometry using the Nanodropt 200 spectrophotometer (Fischer Scientific, Madrid, Spain) at 260 nm. Only extractions with a ratio 260/280 nm >1,4 were considered in this study. RNA integrity was evaluated by electrophoresis using the Bioanalizer (Agilent technologies, Santa Clara CA, USA). Only those extractions with an RIN near 10 were used for gene expression studies.

cDNA was synthesized using the TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Reactions were 1/2 diluted and preamplification was carried out using the TaqMan preamp master mix (Applied Biosystems, Foster City, CA, USA) according to the supplier instructions. Assays on demand against PGC-1α, CYTC, SOD, and GAPDH were purchased from Applied Biosystems and gene expression was carried out in a 7900HT real-time thermocycler (Applied Biosystems, Foster City, CA, USA). The comparative ΔC_t method was used to calculate relative expression levels of the genes included [23].

2.3. Determination of Total Antioxidant Status (TAS). TAS was determined in plasma samples using the Total Antioxidant Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions. This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]) to ABTS+ by metmyoglobin. Capacity of antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analog. Results are expressed as mM Trolox equivalents.

2.4. Analysis of Glutathione Peroxidase (GPX) Activity. GPX activity was estimated in plasma samples using the GPX assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to supplier instructions. Plasma samples were 1/2 diluted in sample buffer (provided with the kit) and the GPX activity was evaluated calculating the change in absorbance at 340 nm (ΔA_{340} nm/min) as it is described in the user's manual included in the kit. Results are presented as nmol/min/mL.

2.5. Data Analysis. Data are presented as the mean ± SEM. Statistical analysis of the results was carried out by nonparametric Mann-Whitney test and nonparametric Spearman correlation analysis using the GraphPad software (GraphPad Software Inc., San Diego, CA). Significance was accepted when $P < 0.05$.

Table 1: Clinical, molecular, and biochemical features of IPAH patients.

ID	Age (years)	Sex	PaO_2 (mmHg)	6 MWT (m)	PAP (mmHg)	CI (L/min/m^2)	PVR (dyn/sec/cm^2)	VR	PGC-1α RE	CYTC RE	SOD RE	TAS (mM)	GPX (nmol/min/mL)	Treatment
HP1	45	F	67	595	48	1.3	12.4	No	2.87	2.45	4.12	0.21	70.23	e + b
HP2	75	M	62	255	70	1.8	11	No	0.34	0.42	0.24	0.05	23.76	a + s + i
HP3	34	F	85	554	35	2.8	6.2	YES	67.00	38.50	7.45	0.28	119.05	n
HP4	58	F	70	380	48	2.2	12	No	6.07	5.47	2.29	0.19	67.89	b + t
HP5	38	F	83	450	38	3.24	6.1	YES	8.96	6.95	26.77	0.15	91.27	n
HP6	63	F	68	360	40	2.1	8.5	No	3.22	2.59	4.64	0.25	85.91	i + b + s
HP7	64	M	60	240	53	2.3	7.7	No	0.34	0.48	0.14	0.04	20.22	b + s + t
HP8	66	M	57	334	50	1.8	8.6	No	1.41	1.01	0.13	0.11	45.42	s + i + b
HP9	60	F	58	120	49	1.1	18.9	No	0.34	0.08	0.13	0.02	24.26	s + i + b
HP10	55	F	63	320	31	2.5	7.5	YES	7.76	8.34	3.47	0.22	90.85	b + s
HP11	48	F	62	534	59	1.4	13.6	No	0.33	0.36	0.17	0.06	19.21	e + s + b
HP12	72	F	60	280	65	2.2	12.5	No	0.22	0.21	0.50	0.01	14.34	i + b

PaO_2: partial pressure of oxygen in arterial blood, 6MWT: 6-minute walk test, PAP: pulmonary arterial pressure, CI: cardiac index, PVR: pulmonary vascular resistance, VR: vasoreactivity, PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-α, RE: relative mRNA expression, CYTC: cytochrome c, SOD: superoxide dismutase, TAS: total antioxidant status, GPX: glutathione peroxidase, e: epoprostenol, si: sildenafil, i: iloprost, b: bosentan, t: treprostinil, and n: nifedipine.

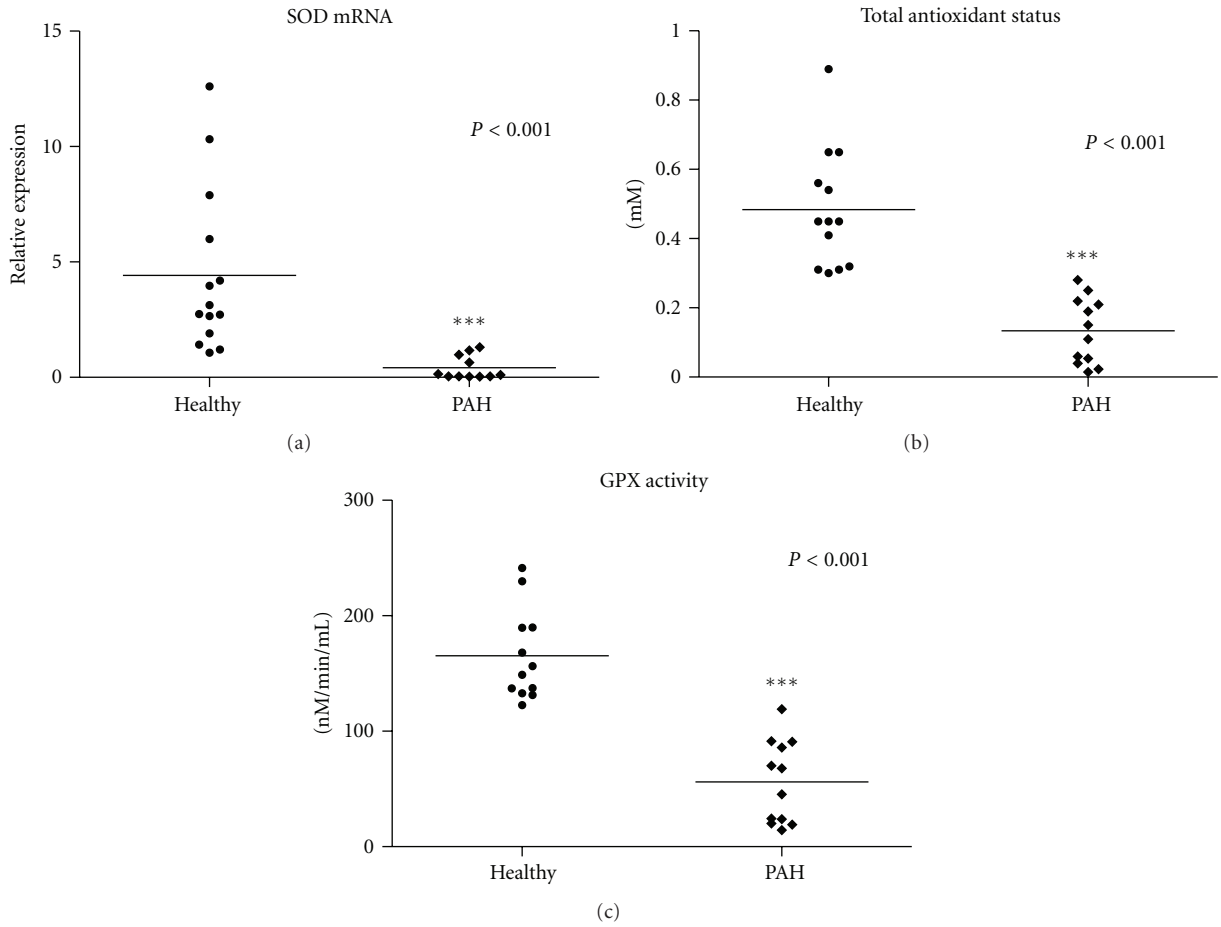

Figure 1: Oxidative status of idiopathic pulmonary hypertension patients (IPAH). Relative expression of SOD (a), TAS (b), and glutathione peroxidase (GPX) activity were evaluated in peripheral blood from 12 IPAH patients and 15 healthy donors. The nonparametric Mann-Whitney test was used to analyze data. Significance was accepted when $P < 0.05$.

3. Results

3.1. PGC-1α CYTC Are Expressed in PAH Blood Samples. Our first objective in this study was to evaluate if the expression levels of PGC-1α and CYTC mRNA were differentially expressed in blood of PAH-diagnosed patients compared to healthy volunteers. Gene expression analysis was undertaken and results indicated that neither of the genes were expressed in healthy donors, contrary to what happened in PAH patients, in which the expression levels of both genes were clearly detected. In order to obtain comparative data, the average ΔC_t of PAH group was calculated and expression levels of each patient were estimated. Results are represented in Table 1.

3.2. Superoxide Dismutase (SOD) Expression Is Reduced in PAH Patients. SOD expression levels were measured in PAH patients. Our results indicate that the expression of this enzyme is lower in PAH patients compared to healthy volunteers (1.01- ± 0.61- and 3.93- ± 0.89-fold change, resp.) as it is represented in Figure 1(a). Next we calculated the relative expression of this gene in a similar way to PGC-1α and CYTC in order to correlate its expression levels with indicators of progression of the diseases. The results obtained are shown in Table 1.

3.3. Total Antioxidant Status Is Reduced in PAH Patients. Hypoxia is one of the most important factors affecting PAH patients. Due to the relevance of PGC-1α induction in the responsiveness against the oxidant injury, we decided to analyze the TAS in PAH patients included in this study. On one hand, we found that TAS is lower in PAH patients compared to healthy donors (0.13 ± 0.027 and 0.484 ± 0.048 mM, resp.), as it is shown in Figure 1(b). On the other hand, we found a clear correlation of TAS levels and the relative expression levels of PGC-1α as described in point 3.5. TAS levels of PAH patients are summarized in Table 1.

3.4. PAH Patients Activity of Glutathione Peroxidase (GPX) Is Decreased. The activity of GPX enzyme in plasma samples of PAH patients and healthy donors was done. Results obtained are represented in Figure 1(c) and clearly demonstrate that the activity of this enzyme is reduced in PAH patients

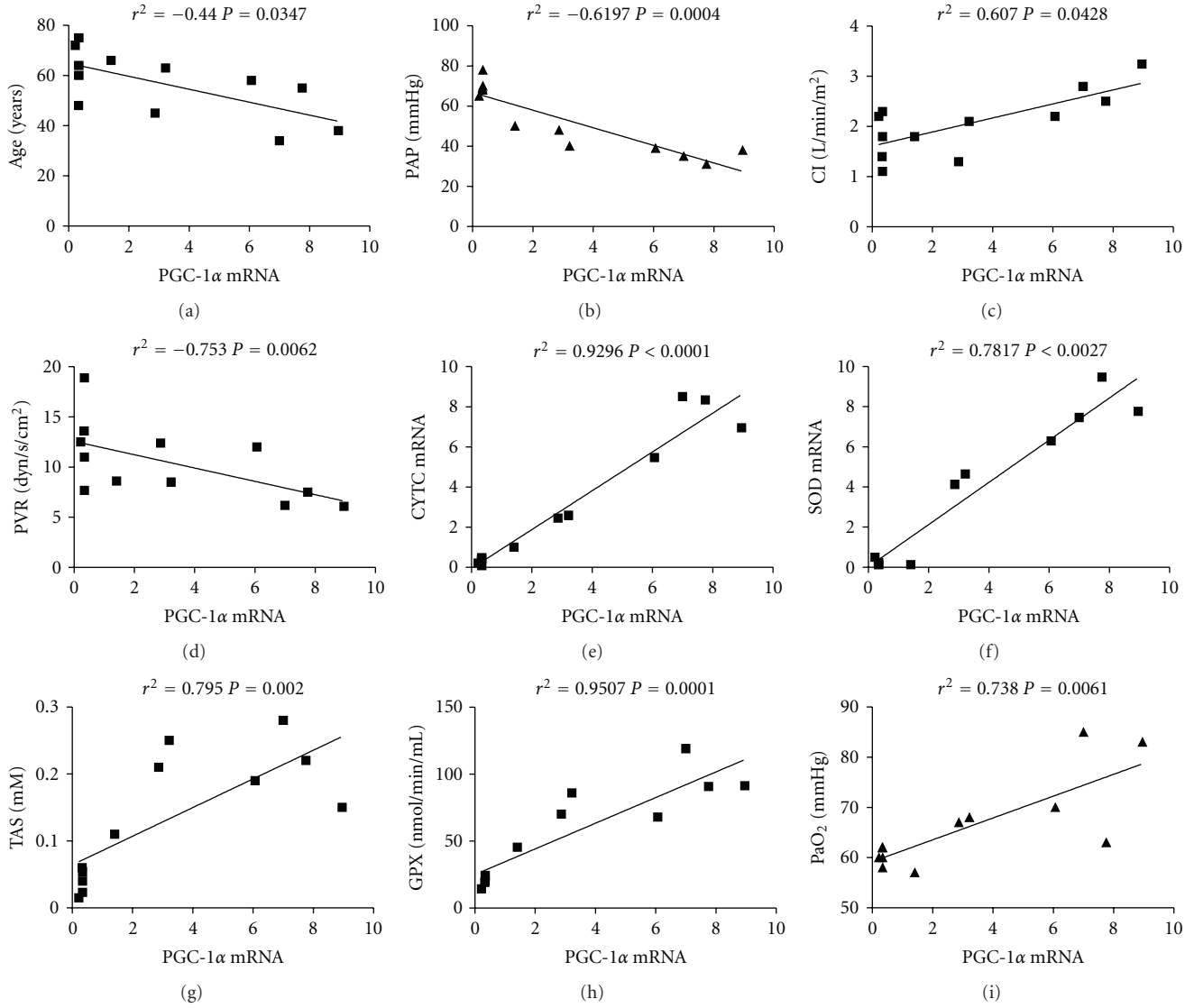

Figure 2: Correlation of PGC-1α with clinical, molecular, and biochemical features. 12 IPAH patients and 15 healthy donors were included in the analysis. The nonparametric Spearman test was used to analyze data. Significance was accepted when $P < 0.05$.

compared to healthy volunteers (56.034 ± 10.37 and 165.46 ± 11.38 nM/min/mL, resp.). GPX activity is summarized in Table 1.

3.5. Multiple Regression Analysis of Clinical, Molecular, and Biochemical Features of IPAH Patients. Finally, multiple-regression analysis of clinical, molecular, and biochemical data was done using the nonparametric Spearman test. The correlation matrix is shown in Table 2. Concerning PGC-1α, results are summarized in Figure 2. We found a negative correlation between age, PAP, and PVR (Figures 2(a), 2(b), and 2(d)). This correlation was significant for PAP and PVR ($P = 0.0001$ and $P = 0.0426$, resp.) but not for the age ($P = 0.108$). On the contrary, the correlation was positive for CI, PaO_2, CYTC and SOD relative expression, TAS, and GPX activity (Figures 2(c), 2(e), 2(f), 2(g), 2(h), and 2(i)). In all cases the change was considered significant. No correlation was found between PGC-1α levels and 6 MWT, which was only significantly correlated with age of the patients.

4. Discussion

In this study we analyzed the expression levels of PGC-1α, CYTC and SOD mRNA as well as TAS and GPX activity in peripheral blood of 12 well-characterized IPAH-diagnosed patients and 15 healthy donors. Our results demonstrate that mRNA of PGC-1α and CYTC can be detected by real time RT-PCR in PAH patients, but not in healthy volunteers. On the other hand, relative expression levels of SOD are decreased in these patients, compared to controls, as well as TAS and GPX activity. Correlation studies carried out indicate that there is a clear correlation between PGC-1α levels and the clinical parameters included in this study, indicating the progression of the disease and the oxidative

Table 2: Multiple-regression analysis of clinical, molecular, and biochemical features of IPAH patients.

	PGC-1α	6 MWT	Age	PAP	CI	PVR	CYTC	SOD	TAS	GPX	PaO_2	
PGC-1α		0.15585	0.03470	0.00041	0.04289	0.00625	0.00012	0.00277	0.00206	0.00010	0.00618	
6 MWT	0.15585		0.00451	0.07089	0.70377	0.55674	0.06251	0.12445	0.01532	0.09516	0.01279	
Age	0.03470	0.00451		0.04786	0.35841	0.31914	0.03581	0.04786	0.03317	0.01683	0.01097	
PAP	0.00041	0.07089	0.04786		0.00791	0.00824	0.00000	0.00033	0.00136	0.00095	0.00705	
CI	0.04289	0.70377	0.35841	0.00791		0.00053	0.01155	0.01412	0.26858	0.10484	0.06642	
PVR	0.00625	0.55674	0.31914	0.00824	0.00053		0.00136	0.04461	0.05484	0.01025	0.07415	*P* values
CYTC	0.00012	0.06251	0.03581	0.00000	0.01155	0.00136		0.00136	0.00015	0.00008	0.00209	
SOD	0.00277	0.12445	0.04786	0.00033	0.01412	0.04461	0.00136		0.01391	0.00736	0.00038	
TAS	0.00206	0.01532	0.03317	0.00136	0.26858	0.05484	0.00015	0.01391		0.00033	0.00401	
GPX	0.00010	0.09516	0.01683	0.00095	0.10484	0.01025	0.00008	0.00736	0.00033		0.00554	
PaO_2	0.00618	0.01279	0.01097	0.00705	0.06642	0.07415	0.00209	0.00038	0.00401	0.00554		
PGC-1α		0.43663	−0.61973	−0.87326	0.60071	−0.75354	0.92960	0.78171	0.79579	0.95073	0.73852	
6 MWT	0.43663		−0.75524	−0.53846	0.12281	−0.18881	0.55245	0.46853	0.67832	0.50350	0.69123	
Age	−0.61973	−0.75524		0.58042	−0.29123	0.31469	−0.60839	−0.58042	−0.61538	−0.67133	−0.70176	
PAP	−0.87326	−0.53846	0.58042		−0.72281	0.72028	−0.94406	−0.86014	−0.81119	−0.82517	−0.72983	
CI	0.60071	0.12281	−0.29123	−0.72281		−0.84562	0.69825	0.68421	0.34737	0.49123	0.54577	
PVR	−0.75354	−0.18881	0.31469	0.72028	−0.84562		−0.81119	−0.58741	−0.56643	−0.70629	−0.53334	Correlation coefficients
CYTC	0.92960	0.55245	−0.60839	−0.94406	0.69825	−0.81119		0.81119	0.88112	0.89510	0.79299	
SOD	0.78171	0.46853	−0.58042	−0.86014	0.68421	−0.58741	0.81119		0.68531	0.72727	0.85615	
TAS	0.79579	0.67832	−0.61538	−0.81119	0.34737	−0.56643	0.88112	0.68531		0.86014	0.76141	
GPX	0.95073	0.50350	−0.67133	−0.82517	0.49123	−0.70629	0.89510	0.72727	0.86014		0.74386	
PaO_2	0.73852	0.69123	−0.70176	−0.72983	0.54577	−0.53334	0.79299	0.85615	0.76141	0.74386		

PaO_2: partial pressure of oxygen in arterial blood, 6 MWT: 6-minute walk test, PAP: pulmonary arterial pressure, CI: cardiac index, PVR: pulmonary vascular resistance, VR: vasoreactivity, PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-α, RE: relative mRNA expression, CYTC: cytochrome c, SOD: superoxide dismutase, TAS: total antioxidant status, GPX: glutathione peroxidase.

status of patients. We found a negative correlation between expression levels of PGC-1α and age, PAP and PVR, as well as a positive correlation with CI, indicating that those patients with higher levels of PGC-1α have an improvement of lung and heart functions. As pointed out in the results section, no correlation was found between 6 MWT and PGC-1α levels. A possible explanation is that this parameter is affected by other factors like the age of the patients [24].

PGC-1α is a transcriptional coactivator which has been shown to activate a broad range of transcription factors and to regulate genes encoding mitochondrial proteins including CYTC under hypoxemia, as it has been shown in animal models [25–27]. Results presented here demonstrate a good correlation between expression levels of PGC-1α and CYTC in circulating blood of IPHA patients, supporting these findings.

IPAH is characterized by a sustained hypoxia situation that leads to cellular damage and contributes to RV failure [9, 10]. TAS determines the response capacity of a biological system to oxidative-mediated events. This status represents the balance between oxidant and antioxidant molecules. We found a significant decrease of TAS in IPAH patients compared to healthy donors. Despite the coherence of the results obtained, under our knowledge, this is the first study demonstrating this decrease in IPAH patients.

One of the most important antioxidant enzymes is SOD and its expression is reduced under chronic hypoxia [28]. These findings are consistent with the decrease of SOD observed in PAH patients compared to healthy volunteers. SOD over expression prevents the development of PH and ameliorates established PH in hypoxia-induced pulmonary hypertension in mice and primary human endothelial cells [29]. We observed a good correlation between PGC-1α and SOD expression levels, which could support the implication of this antioxidant enzyme in the pathogenesis of IPAH.

Another key enzyme controlling oxidative damage is GPX as it has been observed in lung of IPAH patients [30]. Similar to what happens with SOD, we observed a significant decrease of the activity of this enzyme in IPAH patients compared to healthy donors and a positive correlation with the expression levels of PGC-1α, which is coherent with the antioxidant role of this transcriptional coactivator in oxidative enzymopathies [16].

All patients included in this study are being treated with different combinations of drugs including calcium channel blockers, endothelin receptor antagonists, PDE5 inhibitors and synthetic analogues of prostacyclin. Although the effects of the treatment attenuating the oxidant level are well known [31–33], heterogeneity on treatments linked to the limited number of patients included in this study make difficult to reach a conclusion about the effect of medication on altering the antioxidant/oxidant status. However, regardless of the treatment, all patients considered in this study are under mild/moderate hypoxemia. More studies including the novo patients and comparing the situation before and after medication are needed to understand the effects of treatment on hypoxemia status and the relation with mechanisms controlled by PGC-1α.

The principal limitation of this study is the number of patients included, however IPAH is a rare disease with a prevalence of 2-3 per million per year [34]. Data presented here indicate the possible role of PGC-1α in controlling the progression of the disease and the oxidative status of IPAH patients. This study suggests that the monitoring of circulating PGC-1α mRNA levels could be indicative of the progression of the disease, providing valuable information of parameters like the treatment efficacy and the severity of the progression of the disease. Another important aspect is that the monitoring of circulating PGC-1α involves noninvasive procedures without risk for the patient and that it could be carried out in a fast and economical way. For these reasons we proposed PGC-1α as a potential new biomarker of the progression of PAH.

Acknowledgments

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Review Article

Lung Oxidative Damage by Hypoxia

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One of the most important functions of lungs is to maintain an adequate oxygenation in the organism. This organ can be affected by hypoxia facing both physiological and pathological situations. Exposure to this condition favors the increase of reactive oxygen species from mitochondria, as from NADPH oxidase, xanthine oxidase/reductase, and nitric oxide synthase enzymes, as well as establishing an inflammatory process. In lungs, hypoxia also modifies the levels of antioxidant substances causing pulmonary oxidative damage. Imbalance of redox state in lungs induced by hypoxia has been suggested as a participant in the changes observed in lung function in the hypoxic context, such as hypoxic vasoconstriction and pulmonary edema, in addition to vascular remodeling and chronic pulmonary hypertension. In this work, experimental evidence that shows the implied mechanisms in pulmonary redox state by hypoxia is reviewed. Herein, studies of cultures of different lung cells and complete isolated lung and tests conducted *in vivo* in the different forms of hypoxia, conducted in both animal models and humans, are described.

1. Introduction

Lung's main function is the exchange of gases, hence, it is the organ which makes contact with the higher pressure of oxygen in our body. This is especially significant in the light of the known toxic effect of this gas, although there are precedents of lung malfunction under low oxygen pressure conditions or hypoxia.

The generation of hypoxia occurs when staying at high-altitude environments or when receiving mixtures of contaminated gases. Thus, this condition is also a factor in various lung pathological processes like in obstructive sleep apnea (OSA), acute lung injury, asthma attacks, atelectasis, chronic obstructive pulmonary disease, and idiopathic pulmonary hypertension. Lung hypoxia is related, in acute form, to the increase of the pulmonary artery pressure [1], epithelial malfunction [2, 3], edema [4, 5], and lung inflammation [6, 7]. Chronic hypoxia is related to vascular proliferation [8], increase of vascular reactivity [9], chronic pulmonary hypertension, and right heart failure [10–12]. Different lung diseases have identified the participation of reactive oxygen species (ROS) in their pathogenesis [13–16].

Historically, the study of oxidative damage has been linked to the increase of O_2 content in both environment and organs; one of the first times where the oxidative damage of an organ was probably studied may have been when the effect of hyperoxia on the lung tissue was reported [17–19]. Logic tells us that there is a need of O_2 presence for the generation of ROS and oxidative damage; likewise, it would be unlikely that oxidative damage occurs when this element is less available [20]. Currently, this paradigm has been changed by identifying hypoxia as the generator of ROS and oxidative damage for systems as well as specific organs. Although oxidative damage associated with hypoxia on the organism has been the subject of several studies [21–24], the knowledge of the effects on lung tissue is relatively poor, particularly in humans. The latter is probably because of the difficulty in obtaining samples of this organ.

The decrease of pO_2 is monitored by the pulmonary arteries smooth muscle cells (PASMCs); these cells react

to hypoxia favoring bronchial vasoconstriction [25]. An opposite effect occurs in the systemic circulation [26], where hypoxia favors vasodilatation of both arteries and veins [27, 28]. Pulmonary arterial vasoconstriction caused by hypoxia appears to be more effective when hypoxia is present in alveolar lumen regarding this stimulus in the lumen of arterioles and venules. This response to the decrease of alveolar pO_2 in ventilation (V) attempts to improve the diffusion of O_2 into the blood through changes in perfusion (Q), a specific feature of the lungs [29]. Alterations in the V/Q relationship will trigger compensatory hypoxic vasoconstriction, which will attempt to normalize the V/Q relationship [29]. This effect will be accompanied by an increase in alveolar pCO_2 and respiratory acidosis [29]. In this context, the maximum steady state of hypoxic pulmonary vasoconstriction occurs between 25 and 50 mmHg of alveolar pO_2; therefore, the blood redistribution is maximum at these levels. However, the magnitude of hypoxic pulmonary vasoconstriction can decrease with levels <25 mmHg resulting in a reduced blood flow redistribution, deteriorating the V/Q, and worsening hypoxemia [26]. This phenomenon is favored with the existence of a lung disease or exposure to altitude. Now, the increasing of ROS in hypoxic lung has an important role in stimulating PASMC contraction in addition to enhancing inflammation and to establishing oxidative damage as the case may be. This will depend on the severity and duration that hypoxia has with a wide range of effects from the absence of tissue damage to the dysfunction on epithelial and muscle cells associated with vascular remodeling and proliferation (see Figure 1). Current evidence suggests that the primary sensor of hypoxia for the development of pulmonary vasoconstriction is the PASMC mitochondria, which increases the production of ROS at low pressures of O_2, probably in the complex III of the electron transport chain. It is possible that there are secondary sensing mechanisms that contribute to this effect, which will increase the production of ROS during hypoxia such as sarcolemmal NADPH oxidasefrom pulmonary vasculature. Researchers have demonstrated an increase in the mitochondrial ROS generation in various tissues in response to hypoxia, including PASMC. The probability that lower concentrations of oxygen in lungs are sufficient to produce similar effects to those observed in other tissues at higher concentrations is true [26].

In this paper, the proposed mechanisms that explain the redox state changes induced by hypoxia are described (see Figure 1). Hypoxia is herein approached, in its different forms, as for exposure time and its varieties: normobaric, hypobaric, hypercapnic hypoxia or in the ischemia and reperfusion process. Furthermore, pharmacological substances [30–32] and measures (transgenic animals) that have been used to describe the participation of ROS in pulmonary function abnormalities for this condition are included, as well as in the search for strategies to mitigate the effects of the hypoxic pulmonary oxidative damage (see Figure 2). This paper also includes protocols of hypoxia performed in different types of lung cells, homogenized lung cells, pieces of tissue, vascular rings, experimental model of isolated-perfused lung, use of KO animals and noninvasive methods

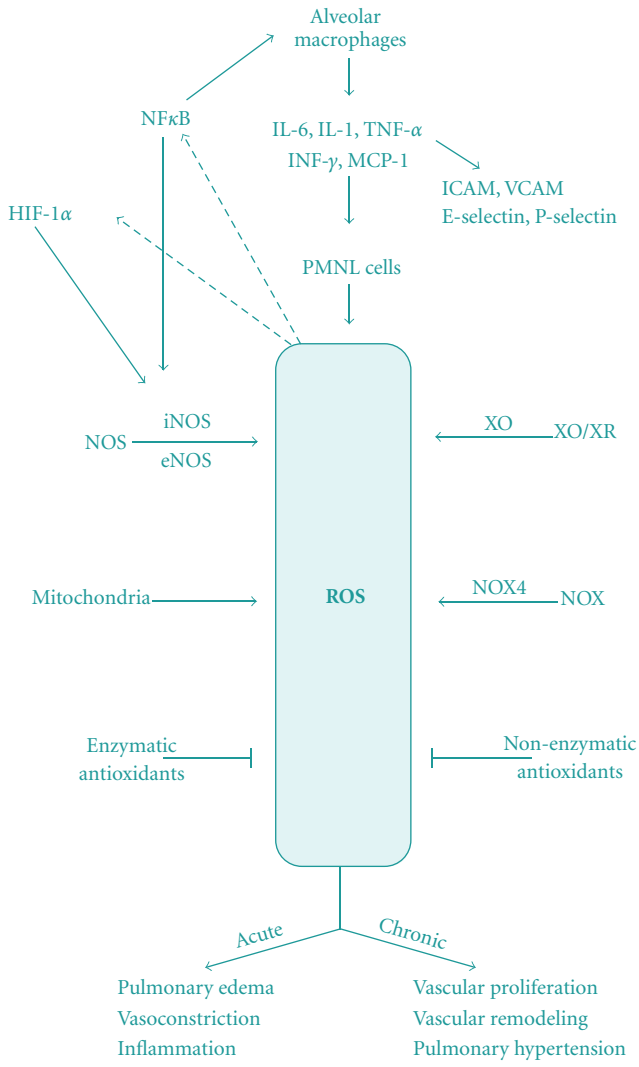

Figure 1: Proposed mechanisms (and effects) for the ROS generation in lungs exposed to hypoxia.

such as the analysis of directly exhaled air and exhaled breath condensate (EBC).

2. ROS

Living under aerobic conditions involves the formation of ROS. These substances have among their more commonly formed constituents superoxide, hydrogen peroxide, hydroxyl, and peroxyl, as well as nitric oxide and peroxynitrite. Some of these ROS contain unpaired electrons (free radicals) and may damage the different types of biomolecules that constitute us: lipids, proteins, carbohydrates, and nucleic acids. The interaction of biomolecules with ROS may occur in the physiological context as well as promoting cellular malfunction, so they have been involved with different diseases [33, 34]. The formation of ROS in the hypoxic context has been a subject of wide-ranging debate in both theory and reported results; the latter is probably influenced by used experimental models and methods by which ROS

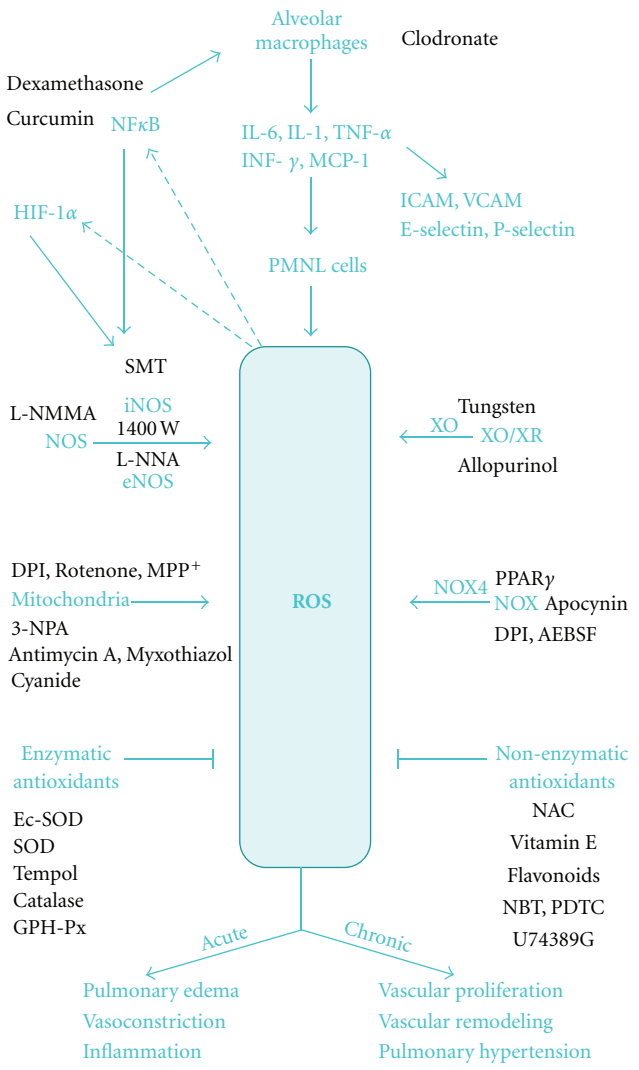

Figure 2: Inhibitors for ROS generation and antioxidants used to study the pulmonary oxidative damage by hypoxia. *NOX inhibitors*: diphenyleneiodonium (DPI) [39, 71–73, 91, 207]; peroxisome proliferator-activated receptor (PPARγ) [79]; 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) [74]; apocynin [42, 207]. *NOS inhibitors*: N^G-monomethyl-L-arginine (L-NMMA): inhibitor of the three isoforms [31]; N-(3-(aminomethyl)benzyl) acetamidine (1400 W) and S-methylisothiourea sulfate (SMT): inhibitors of iNOS [31, 59]; L-NG-nitroarginine (L-NNA): inhibitor of eNOS and nNOS [59, 182]. *Mitochondrial inhibitors*: complex I: rotenone [38, 40]; diphenyleneiodonium (DPI) [39, 71–73, 91], 1-methyl-4-phenylpyridinium (MPP^+) [75]. Complex II: 3-nitropropionic acid thenoyltrifluoroacetone: (3-NPA) [208]. Complex III: antimycin A [38, 40, 208]; myxothiazol [40, 42]. Complex IV: cyanide [40]. *Enzymatic antioxidants*: Catalase [40, 45, 51, 91, 209]; SOD [37, 49, 91]; Ec-SOD [90]; glutathione peroxidase [49]. *Nonenzymatic antioxidants*: N-acetylcysteine (NAC) [89, 209]; vitamin E [4]; flavonoids [186]; nitro blue tetrazolium (NBT) [30–32]; pyrrolidine dithiocarbamate (PDTC) [42, 209]; U74389G [91]. *XO/XD inhibitors*: allopurinol [89]; tungsten [85]. *Others*: clodronate, acts by decreasing the number of macrophages.

have been determined. Evidence of oxidative damage by hypoxia, in the lungs, is consistent; however, the mechanisms about how it is produced are still being discussed. In this regard, several sources for the generation of ROS in hypoxia have been proposed as detailed below.

3. Mitochondria

This organelle is one of the sites of constant ROS production, particularly superoxide anion. This process specifically occurs in the mitochondrial electron transport chain (ETC), in complexes I and III [35, 36]. Some early reports showed a decrease in intracellular ROS formation by hypoxia, hence, Paky et al. [37], found a decrease in ROS formation and those came from the intracellular environment when the lucigenin assay was applied to a preparation with isolated perfused lung in anoxia (95% N_2 and 5% CO_2). Archer et al. [38] found an ROS decrease in isolated and perfused lungs by using luminol, when 2.5% O_2 during 6 min was applied. The same result was found when rotenone and antimycin A were applied, both ETC inhibitors in complexes I and III, respectively. A similar result was found in rat aortic rings at 36 mmHg pO_2, by using lucigenin [39]. In pulmonary artery rings, endothelium-denuded preparations were carried out and a decrease in ROS formation regarding normoxia (analyzed by lucigenin, 2′,7′-dichlorodihydrofluorescein diacetate (DCF), and Amplex Red) in hypoxia (40 mmHg PO_2) was found when rotenone and antimycin A were applied. This effect was not observed when cyanide was applied, an inhibitor of mitochondrial complex IV [40].

Another group of investigators have reported increases in ROS production in hypoxia. Grishko et al. [41], in cultures of pulmonary artery endothelial cells (PAECs), incubated at 25 mmHg O_2 for 15 min and found ROS increases with DCF. Waypa et al. [42] found an ROS increase in pulmonary artery smooth muscle cells (PASMCs) exposed to 2% O_2; this increase was inhibited by myxothiazol (blocker of mitochondrial complex III). Similar results were found by other authors; however, the use of lucigenin and DCF was questioned with regard to its properties to determine ROS properly [43]. Probably the use of more specific methodologies to measure ROS in the last few years, based on proteins that are sensitive to oxidation like HSP-FRET and roGFP, weighed the balance towards hypoxia, as a condition where ROS and oxidation increase [44–48]. Therefore, a decrease of the GSH/GSSG ratio was observed in cultures of PASMCs exposed to 1.5% O_2, which was inhibited by myxothiazol. Moreover, increase of cytoplasmic oxidation by hypoxia was found using HSP-FRET. This change of oxidation by hypoxia was inhibited by nonenzymatic antioxidants like pyrrolidine dithiocarbamate and N-acetylcysteine (NAC). The same effect was gained with the overexpression of mitochondrial catalase through a viral vector. Overexpression of SOD and the use of cyanide did not change the ROS increase by hypoxia [49]. By using the roGFP protein indicator in PASMCs, Waypa et al. [50] after administering 1.5% O_2 for 30 min found ROS increases in the mitochondrial intermembrane space and at a cytoplasm level, and ROS decreases in the mitochondrial matrix. Overexpression in these cells to cytoplasmic catalase

inhibited the ROS increases in this compartment [50]. In a subsequent report using rat lungs slices, Desireddi et al. [51], found an increase in cytosolic ROS by hypoxia (1.5% O_2). This increase was attenuated by the catalase overexpression induced by viral vectors [51]. The complete description of the ROS generation at a mitochondrial level was recently revised by Schumacker [52].

4. Nitric Oxide Synthase (NOS)

This enzyme is capable of forming free radical nitric oxide from L-arginine. This molecule has been involved in multiple processes both physiological (vasodilation and bronchodilation) and pathological (inflammation and oxidative damage) in lungs [15, 53]. In addition, it has been used as a drug [54]. NOS is presented in three isoforms, endothelial (eNOS), neuronal (nNOS), and inducible or iNOS. The expression of the two first ones depends on the activity of the calcium/calmodulin system, whereas the expression of iNOS is mainly stimulated in the context of an inflammatory process. Another recognized stimulating factor to the expression/activity of this enzyme is hypoxia. Xue et al. [10] found the increase of the expression of NOS in endothelium of small pulmonary vessels and vascular smooth muscle after exposing rats during 2 to 4 weeks at a 10% O_2. Shaul et al. [55] found increases of the expression of the nNOS and eNOS after 7 and 21 d of hypoxia, while Fagan et al. [56] described increases of the expression of eNOS and iNOS in lung homogenates of rats exposed during 6 weeks at 5,200 m. Probably in hypoxia the more active isoform that is related to vascular remodeling, observed in pulmonary hypertension, corresponds to the iNOS, which in normoxia has a poor role in the production of NO; however, it is increased in hypoxia as previously suggested [10, 57].

Rus et al. [58] subjected rats to hypobaric hypoxia at a barometric pressure of 225 mmHg during 30 min and subsequently to reoxygenation times from 0 to 5 d, finding in lung homogenates higher oxidative damage to proteins, lipids, and apoptosis increase. The administration of a selective inhibitor of iNOS, the most abundant isoform in the airways, N-(3-(aminomethyl) benzyl) acetamidine (1400 W), resulted in a decrease of nitrite and nitrate, besides decreasing the lipid peroxidation and oxidative damage to proteins. The inhibition of iNOS by 1400 W in a ventilated and isolated perfused lung model of rabbits implicated the decrease in the pulmonary artery pressure and a lower filtration of the pulmonary capillaries in a hypoxic (3% O_2) and hypercapnic (11% CO_2) environment, while the use of L-NG-nitroarginine (L-NNA) inhibitor of the eNOS and nNOS did not produce changes of vascular tone under this conditions [59].

In a model of rats subjected to hypoxia (10% O_2) and hypercapnia (6.5% CO_2) an increase of iNOS (in both mRNA and proteins) was found. In addition, lower quantities of mRNA, proteins and activity of soluble guanylate cyclase (NO effector) were found. This process is probably involved in the development of pulmonary hypertension by hypoxia; this report may be significant in those pathologies where these changes of gases' pressures coexist like in chronic obstructive pulmonary disease and sleep apnea.

5. NADPH Oxidase (NOX)

Its function is to form free radicals, particularly superoxide; the formation of this free radical begins with the transfer of electrons from NADPH to molecular oxygen [60–62]. This enzyme has a great functional relevance since it decreases the levels of nitric oxide when it reacts with it and forms peroxynitrite [63]. There are NOX isoforms, from 1 to 5, and DUOX 1 and 2 [61, 64]. From the structural composition point of view, lung tissue is formed by several cell types that give rise to the presence of more than one variety of NOX in this organ; endothelial cells express NOX1 and NOX5 but predominantly NOX2 and NOX4. Alveolar macrophages express NOX2, fibroblasts and vascular smooth muscles express NOX4, ciliated epithelial cells of the airways express DUOX1 and DUOX2, and type II alveolar cells express NOX1 [65, 66]. The expression of the different NOX isoforms in cells can be modified according to the conditions they are exposed to just as in the case of the increase of NOX4 in some tumor tissues [67]. Notwithstanding to a greater or lesser extent any of the isoforms present in the described cell types can participate by forming free radicals, and, in the case of lung tissue, it is known that the major source of free radicals derived from endothelium is originated in the NOX [68, 69].

PAECs obtained from bovines were exposed to a protocol of ischemia *in vitro* (24 h at a 3% O_2) in which increases of extracellular hydrogen peroxide were found. The production of this prooxidant was inhibited by diphenyleneiodonium (DPI) [70]. Marshall et al. [71] obtained, in rings of pulmonary arteries of cats, that the application of DPI favored the relaxation of hypoxic vasoconstriction induced by three series of gases in hypoxia (95% N_2, 5% CO_2). Thompson et al. [72], in pieces of rats pulmonary arteries, found that in one hour of hypoxia (95% N_2, 5% CO_2) an increase of the pressure within these vessels was observed and that this phenomenon was diminished by the application of DPI. Jones et al. [73] found that a relaxation of arteries was produced by DPI, in a similar preparation of rats arteries, to the same percentages of gases supplied in the previous study.

In preparations of isolated-perfused lungs of rabbits, exposure to series of 10 min at 3% of O_2, application of 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), an inhibitor of the NADPH oxidase, resulted in a decrease of hypoxic vasoconstriction [74]. Archer et al. [39] found a large decrease of radicals' generation in perfused lungs of rats that lost the $gp91^{phox}$ subunit, after applying 2.5% O_2 during 30 min; however, the maintenance of the vasoconstrictor phenomenon to hypoxia was observed.

Weissmann et al. [75] reported in perfused, ventilated, and isolated lungs a decrease of the release of superoxide radicals measured by spin resonance by using the 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine marker in deficient mice in $p47^{phox}$ subunit; however, when they were exposed for 30 min at 1% of O_2, an increase of superoxide in the perfusion solution was observed.

In models of chronic exposure, it has been reported that in rats exposed at 10% O_2 during 21 d an increase of NOX4 mRNA was found, while no differences for NOX2 mRNA were observed [11].

In a model of rats exposed during 3 weeks at 10% O_2, an increase of the production of superoxide from intrapulmonary artery rings was found, which was related to $gp91^{phox}$ subunit of NADPH oxidase. In addition, there was vascular remodeling and pulmonary hypertension that were not observed in KO rats for $gp91^{phox}$ subunit [76]. Rats were exposed to simulated altitude in hypobaric chamber at 380 mmHg during 21 d where an increase in the ROS' production and an increase in the vascular reactivity of intrapulmonary vessels segments were found; these results were not observed in KO rats for $gp91^{phox}$ subunit [9].

Ismail et al. [8] in culture of human PASMCs found an increase of NOX4, H_2O_2, and cell proliferation that was mediated by the beta type transforming factor when these cells were subjected at 1% O_2 during 72 h. In the same cell types and the same protocol of hypoxia, Lu et al. [77] found similar results in the expression of NOX4 and cell proliferation. Under hypoxia conditions, the expression of the transcription factor NF-κB increased and an increase of the association of this factor to the NOX4 promoter was found. These factors were eliminated when rosiglitazone was added, a pharmacological analogue of peroxisome proliferator-activated receptor (PPARγ), substance that has previously demonstrated to decrease the expression of endothelial NOX [78]. Nisbet et al. [79] demonstrated an increase of NOX4 mRNA and protein, besides an increase in the generation of superoxide anion and vascular remodeling with hypertrophy of the pulmonary artery in rats that were exposed to 10% O_2 during 3 weeks. The administration of PPARγ ligand, rosiglitazone, prevented these changes induced by hypoxia. It is likely that this animal model explains, in part, what happens in humans who suffer idiopathic pulmonary hypertension, since normal human PASMCs increased their NOX4 mRNA levels when they were exposed to 1% O_2 during 24 h and lung tissue of patients who suffer idiopathic pulmonary hypertension showed an increase of the expression of this isoform [11].

Nisbet et al. [80] performed a chronic intermittent hypoxia protocol in rats that simulated the desaturations observed in patients with OSA; for this purpose, the O_2 from 21% to 10% every 90 s during 8 weeks was decreased. After this treatment, an increase of the expression of NOX4 and $p22^{phox}$ in lung homogenates was observed. KO rats to $gp91^{phox}$ which were exposed to the simulated OSA protocol, did not show any changes in the expression of NOX4. These findings support the hypothesis that NOX is involved in vascular remodeling by hypoxia.

6. Xanthine Oxidase/Dehydrogenase

This enzyme is present in all endothelial cells of the organism and also in pulmonary vessels, participating in hypoxanthine and xanthine degradation to uric acid [81, 82]. Throughout this process, superoxide and hydrogen peroxide are formed. Dehydrogenase (XD) form, predominant in normal conditions, can be susceptible to oxidation and proteolysis transforming in oxidase form (XO) [83, 84]. In bovine PAECs exposed to 48 h of hypoxia (95% N_2 and 5% CO_2, balanced with ambient air), XO and XD activity increases was found. Also, an increase in superoxide formation to the medium was found, which was decreased by the use of allopurinol and tungsten, both of them inhibitors of the enzyme. Furthermore, enzymatic activity was inversely related to the O_2 pressure they were exposed to [85]. Hassoun et al. [86] found increases in XO activity, using *in vitro* bovine PAECs at 3% O_2 for 48 h. In the same study, a variation of XO/XD expression between species was reported [86]. Afterwards, the same increase of the XO and XO + XD activity at 3% O_2 for 48 h in PAECs *in vitro* was found. In addition, rats were exposed to 380 mmHg for 5 days, finding an increase in XO activity and the XO/XR ratio. The supplementation of L-arginine decreased the XO activity [87]. Hassoun et al. [88] found an increase in XO activity in lung homogenate in rats exposed to 380 mmHg for 24 h. This change was related to the increase of fluid content in the lungs of the animals. Rats exposed to 10% O_2 for 21 days showed an increase in XO activity from the first day that remained throughout the period. Also, greater lipid peroxidation in homogenates was observed. The use of allopurinol avoided the pulmonary hypertension and right ventricular remodeling [89].

After endothelial cells were exposed to hypoxia for 24 h (1% O_2), they showed a great increase in intracellular ROS production measured with CDF and that was related to an increase in XO concentration. In the same experimental series, transfected cells of the same type of cells overexpressing intracellular SOD did not show any increase in the ROS production or changes in the XO amount [90]. In a *in vivo* study, rats were exposed to 10% O_2 for 10 d and an increase in total leucocyte in BALF and in ROS production and XO amount in lung tissue was found. In transgenic rats overexpressing extracellular SOD, no evidence of this phenomenon was found [90]. In a model of chronic exposure to hypoxia (13% O_2) for up to 14 d in newborn rats, vascular proliferation increased and also vascular reactivity increased oxidative injury to lipids and proteins. The administration of allopurinol decreased at the fourth day the increase of XO, consequently decreasing oxidative tissue injury; the same process happened when administering Tempol, an analogous of SOD and U74389G, and a synthetic nonenzymatic antioxidant [91].

7. Lung Inflammation

The inflammatory process is one of the factors involved in the increase of ROS's generation and tissue oxidative damage; in turn, ROS are involved in the NF-κB release, a transcriptional factor that triggers the inflammatory process; HIF1-α has a central role in the response to hypoxia as well [92, 93]. Alveolar hypoxia corresponds to a proven stimulus that triggers inflammation that is first localized and later it becomes systemic; in this point, the alveolar macrophages'

role seems to be essential [6]. It has been previously demonstrated the increase of both the *in vitro* secretion of monocyte chemoattractant protein (MCP-1) and the tumor necrosis factor-alpha (TNF-α) derived from alveolar macrophages of rats exposed to 5% O_2 for 5 h [94]. Gonzalez et al. [95] observed a decrease of the inflammatory response activated by hypoxia after the administration of clodronate liposomes to deplete lung of alveolar macrophages in rats exposed to 10% O_2. In hypobaric hypoxia, an increase in number, proinflammatory activation, and release of ROS by alveolar macrophages has been described [96, 97]. Humans exposed to hypobaric chamber at 4,500 m for one hour during seven days evidenced leukocytosis with a predominance of polymorphonuclear neutrophils leukocytes (PMNLs) on the first day of protocol, while this was not observed on the seventh day. In addition, in *in vitro* PMNLs, which were stimulated by zymosan, the formation of basal superoxide at the end of the seventh day of intermittent altitude was increased and there were no changes in any stage in the formation of ROS by acute exposure [98]. The expression of CD18 and formation of superoxide increased in an exposure to 3,196 m. Training and altitude exposure decreased the capacity to form superoxide by PMNL [99]. One hour at 5% O_2 increased inflammatory cell adhesion to alveolar epithelial cells of rats. Thus, Beck-Schimer et al. [100] found an *in vitro* increase of ICAM-1 and VCAM-1, which was related to a higher PMNL and macrophages adhesion to alveolar cells. In lung homogenates of rats subjected to hypoxia (10% O_2) increases of myeloperoxidase (MPO), NF-κB activity, and mRNA of ICAM-1, VCAM-1, HIF-1α, macrophage inflammatory protein beta (MIP-1β), and MCP-1 were found. In BALF, an increase in both proteins and total number of cells was found. These changes were reduced by the depletion of alveolar macrophages using clodronate [94]. In rats exposed to 9.9% O_2 for 24 h, an increase in the PMNL number and TNF-α, IL-1 beta and IL-6 and MPO was found. At the same time, an increase in the generation of nitrite, nitrate, and hydroxyl radicals in BALF was evidenced. These changes were associated with the increase of the expression of HSP70 in lungs. The hypoxic preconditioning (18.3% O_2 during 5 days) decreased the formation of previously described ROS, nitrites, and, also, pulmonary edema [7]. Shukla et al. [101] exposed rats during 48 h at 7,619 m, observing an increase in ROS formation measured as DCF and lipid peroxidation (TBARs and 4-hydroxynonenal). These changes were associated with increases of TNF-α, interferon gamma and interferon alpha, as well as MCP-1 in BALF. In turn, an increase in mRNA and protein expression of HSP-32 and HSP70 was observed. Furthermore, the expression and activity of HIF-1α increased together with the increase of its targets EPO and GLUT-1. The use of $CoCl_2$, a stabilizer of HIF-1α [102] and an inducer of ischemic preconditioning [103], decreased both oxidative damage and inflammation of this organ by hypoxia.

Exposure of rats during 5 h at 9,142 m favors the generation of pulmonary edema and promotes the release of inflammatory mediators (MCP-1, IL-1, TNF-α, TGF-β, and IFN-γ) and adhesion molecules (ICAM-1, VCAM-1, and P-selectin) in BALF. These changes were associated with increases of NF-κB in lung homogenates. The administration of $CoCl_2$ reduced these effects [5]. Sarada et al. [104] found pulmonary edema and an increase of ROS (DCF), lipid peroxidation (MDA), NF-κB, IL-1, IL-6, and TNF-α and also ICAM, VCAM and P-selectins in lung homogenate of rats exposed at 7,620 m. Preconditioning with curcumin, an inhibitor of the expression of NF-κB, reduced edema and reduced the expression of NF-κB. Rats exposed for 5 h at 9,144 m, in hypobaric chamber, showed an increase of IL-6, IL-10, TNF-α, MCP-1, and VEFG in BALF. The administration of an ethanol extract of *Hippophae rhamnoides L.* leaves, rich in flavonoids, with known antioxidant and anti-inflammatory effect such as quercetin, epicatechin, and flavonols, or the administration of dexamethasone favored inhibition in the formation of proinflammatory factors, reduced edema, and decreased VEGF, the latter, a known promoter of the endothelial permeability increase. An increase of E-selectin after a cicloergometric exercise at 3,810 m has been reported in the BALF analysis in humans [105]. In the same kind of sample, subjects who had pulmonary edema at a moderate altitude (2,600–3,000 m) increased the amount of alveolar macrophages, lymphocytes, PMNL, and total proteins. An increase of IL-1β, IL-6, IL-8, and TNF-α was found; after recovery, these values returned to normal. A similar result was previously reported in four patients for the same cytokines in BALF at a similar height [106].

8. Pulmonary Antioxidant Defenses

The oxidizing effect of ROS can be countered by reducing agents called antioxidants, preventing their formation or removal and, hence, modeling the cellular redox state. Antioxidants can be classified according to their nature as enzymatic or *nonenzymatic* antioxidants. In lungs, the main enzymatic reducers are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and thioredoxin (TRx) [107, 108]. Among the *nonenzymatic* reducers we can find mucin, urate, glutathione (GSH), ascorbate, ceruloplasmin, transferrin, vitamin E, ferritin, and small molecules such as bilirubin [107, 109–113]. Regarding this category, in the central airways in lungs we can find mucopolypeptidic glycoproteins with high molecular weight, released by epithelial cells and glands, which are capable of increasing the amount of mucus. Furthermore, a thin protective film so called epithelial lining fluid (ELF) is deposited on the surface of the airways, which has a significant amount of both enzymatic and nonenzymatic antioxidants among its components [107, 114]. In the ELF, the most important *nonenzymatic* antioxidant contributions are mucin, urate, ascorbate, and GSH; among the enzymatic antioxidants are SOD, CAT, and GSH-Px [114].

9. Enzymatic Antioxidants

SOD has an important role in catalyzing the conversion of superoxide to H_2O_2 and O_2. This enzyme has three isoforms: the copper-zinc SOD (CuZnSOD), located in the nucleus,

cytoplasm, and peroxisomes, the manganese SOD (MnSOD) located in the mitochondria, and the extracellular SOD (Ec-SOD), which is outside of the plasmatic membrane of the lungs [115]. In hypoxia, MnSOD seems to be the target of modifications of this enzymatic variety, unlike the other isoforms. In this regard, Nakanishi et al. [116] found a decrease in the activity of MnSOD in rats' lung homogenates, after 5 d at 5,500 m height in hypobaric chamber. Subsequently, Cantin [117] observed a reduced activity of mitochondrial MnSOD after subjecting rats to 7% O_2 during 60 min. Russell and Jackson [118] observed an increase of the MnSOD protein expression and activity in lung homogenates of rabbits by using an *in vivo* model of hypoxia by lung collapse, during 7 d without subsequent reexpansion. On the other hand, when a group of rats was preconditioned to low concentrations of O_2 simulating a height of 5,500 m during 15 h, an increase of the activity and protein level of MnSOD but not of CuZnSOD was produced [119]. Rusell et al. [120] did not find any changes in MnSOD mRNA in alveolar type II cells after subjecting *in vivo* rabbits' lungs to collapse during 7 d without subsequent reexpansion. Russell et al. [121] did not observe increases in mRNA of mitochondrial MnSOD of the lungs in transgenic rats which overexpressed this enzyme, after being for 7 d at 10% O_2 under normobaria. However, when isolated and ventilated rats' lungs were subjected to subsequent reoxygenation to an anoxia period (10 min to 5% CO_2 and 95% N_2 and then reoxygenated 10 min to 21% O_2), the content of MnSOD mRNA in lung homogenate was increased [122]. These studies confirm the importance of MnSOD as one of the major lines of defense against pulmonary hypoxia. In addition, Ec-SOD isoform is the main extracellular enzymatic antioxidant in lungs [123, 124], with a known protector effect in the lining of the lungs fluids [125, 126] and at interstitial level and a high affinity to the extracellular matrix [127, 128] in rats and humans. In these studies, developed under hyperoxia, fibrosis, and hemorrhage conditions [128–131], the protective effects of Ec-SOD against oxidative damage by hypoxia have also been observed. Both this enzymatic antioxidant and GSH-Px are in charge of protecting the vulnerable targets to hypoxia-induced free radicals, such as alveolar epithelial cells and the endothelium of pulmonary capillaries [125]. Nozik-Grayck et al. [132] studied rats with pulmonary hypertension after chronic hypobaric hypoxia. The group of wild-type rats, after 1 d of exposure at a simulated height of 5,486 m, increased the activity of Ec-SOD and then it decreased at day 35. Transgenic rats that overexpressed Ec-SOD did not show any change in the activity of Ec-SOD and they protected the lung against vascular remodeling and hypertension. In another study, Giles et al. [133] observed a decrease in the expression of Ec-SOD at transcriptional and translational level in lung homogenates of rabbits that were subjected to hypobaric hypoxia (4,572 m for 36 h) at 26 d of gestation. As it happened with Ec-SOD, hypoxia limited the expression of pulmonary MnSOD. A recent study [90] has confirmed the potential inhibitor that the Ec-SOD overexpression has in the processes of pulmonary hypertension induced by hypoxia. According to the authors, the concept of ROS reduction could explain the reversibility of this phenomenon. In vivo, the hypoxic chamber animal model (10 d at 76 mmHg O_2) showed a significant decrease of ROS, right ventricular systolic pressure, and pulmonary vascular wall thickness in the group of transgenic mice that overexpressed Ec-SOD in comparison with the WT adult mice transfected with an empty vector. Other authors have recognized the contribution of Ec-SOD in other oxidative states such as COPD [134], exposure to cigarette smoke [126], and hyperoxia [135]. These results suggest that high concentrations of Ec-SOD present in lung fluids [125] play an essential protective role against the oxidative threat from exposure to external environment.

Catalase is mainly located in peroxisomes of most mammalian cells, but it can also be found in mitochondria and endoplasmic reticulum. This enzyme is responsible for catalyzing the decomposition of H_2O_2 to O_2 and water. In hypobaric hypoxia simulated in chamber, Nakanishi et al. [116] found an increasing trend without significant changes in the activity of CAT in lung homogenates of rats that stayed up to 21 d at 5,500 m. As it happened with MnSOD, Shen et al. [122] demonstrated increases of CAT mRNA subsequent to reoxygenation in a model of isolated lungs of rats subjected to hypoxia and reoxygenation. A recent investigation showed that the activity of CAT was significantly increased with regard to the control in lung homogenate of rats exposed to ischemic preservation. Besides, the increase of CAT was higher as the time of storage increased [136]. Both pregnant and nonpregnant rats exposed to 10% O_2 during 4 d did not show any variation of the activity of CAT [137].

CAT and GSH-Px share the function of controlling the concentrations of H_2O_2; they are the most outstanding cellular mechanisms (to detoxify hydroperoxides) for the neutralization of ROS [138]. Whenever the concentrations of H_2O_2 are in a high level, CAT becomes more effective than GSH-Px and vice versa [117]. Nevertheless, GSH-Px is capable of removing organic peroxides, such as those derived from lipid peroxidation [139]. To control the concentrations of H_2O_2, the enzymatic reaction of GSH-Px uses GSH, which acts as a reducer. Oxidized GSH (GSSG) will be reduced by glutathione reductase (GSH) with the help of NADPH. All glutathione peroxidases described in the body contain selenium, which include the following: the classic cytosolic shape present in all cells (cGSH-Px) [140–143], one associated with membrane phospholipids (PHGSH-Px) [144], other associated with gastro intestine (giGSH-Px) [145] and to extracellular (eGSH-Px) [146]. The latter is part of ELF and it is produced and secreted by alveolar epithelial cells and macrophages increasing lung antioxidant defense against oxidative stress as it occurs during hypoxia [147].

In lungs, antioxidant activity of GSH-Px in ELF is headed by eGSH-Px followed by cGSH-Px. Avissar et al. [147] showed a significant increase of the expression of eGSH-Px and cGSH-Px in a group of bronchial epithelial cell lines and primary human alveolar macrophages when both of them were subjected—*in vitro*—to oxidizing substances such as ozone (O_3). The immediate presence of eGSH-Px in the used medium places these cells and the interstitial cells as potential sources of pulmonary eGSH-Px. In human lungs subjected to oxidizing conditions, as in the case of

smokers, the activity and expression of eGSH-Px in ELF showed some increases [148, 149], the same occurred in asthmatic subjects who were in contact with cigarette smoke [150, 151]. Similar answers have been observed on hypoxia in studies that have quantified pulmonary GSH-Px. In rats placed into a simulated environment at 5,500 m, the activity of GSH-Px in lung homogenate was slightly increased and no significant differences until 21 d of exposure were presented [116]. Likewise, Yeginsu and Ergin [136] published findings about increases of GSH-Px in lungs of rats subjected to 12 h of ischemic preservation in cold solution at 4°C, reaching even lower values than the control group's when preserved at 48 h. On the contrary, Zhao et al. [152] observed a decrease in the activity of GSH-Px in lung homogenate of rabbits exposed to a simulated height at 8,500 m in barochamber during 3 h. Pregnant rats exposed to 10% O_2 during 4 d showed a decrease of the activity of GSH-Px, and no changes in non-pregnant rats were observed [137].

Comhair and Erzurum [146] claim that the transcription of mRNA, under oxidative pathological conditions, protein expression, and release of eGSH-Px to ELF are the most inducible at epithelial level in humans, just as it occurs in the presence of hypoxia.

TRX is a ubiquitous enzyme with a crucial role in controlling cellular redox environment [153]. The active site of this polypeptide has the -Cys-Gly-Pro-Cys-amino acid sequence. Functionally, TRX participates in redox regulation of numerous proteins such as NF-κB, HIF-1α, c-Fos/c-Jun complex, glucocorticoid receptor, estrogen receptor [154]. TRx is induced by the production of H_2O_2 demonstrating a potent cytoprotector effect. Likewise, human TRX (hTRx) protects cells from the increase of superoxide during ischemia-reperfusion (IR) in the reaction that converts hypoxanthine to xanthine by xanthine oxidase [155]. Fukuse et al. [156] observed an increase in the protective capacity of recombinant hTRx *in vivo* in rats of IR in a nonventilated lung during 75 min ischemia. The immediate administration to reperfusion of recombinant hTRx implied higher survival, improved gas exchange, decreased edema, and reduced pulmonary lipid peroxidation [156, 157]. Okubo et al. [158] in a similar protocol of IR administered hTRx, which reduced IR injury in rabbits probably by reduction of ROS. The protective effects in pulmonary IR have been corroborated by several authors. These studies suggest that hTRx could be effective as a radical eliminator in the lung reperfusion treatment, just as it occurs after transplantation [158–160].

10. *Nonenzymatic* Antioxidants

As previously mentioned, there are many *nonenzymatic* antioxidants in ELF that are capable of detoxifying ROS [161]. The latter are the first line of defense against inhaled oxidants from environments such as O_3, nitrogen oxides, and tobacco smoke or against changes in O_2 tension such as hypoxia or hyperoxia. One of them is mucin, which besides acting as an antioxidant regulates the viscosity of mucus [162]. It has been demonstrated that oxidative stress increases the expression and production of mucin, improving the antioxidant condition in the surface of the respiratory tract [163, 164]. Likewise, a decrease of GSH in the respiratory epithelium, just like in hypoxia, will favor the increase of viscosity in mucin. Under oxidative-inflammatory pathological conditions, such as cystic fibrosis and chronic bronchitis, excessive increase of mucus viscosity by production of mucin is considered a negative condition for the airways. Therefore, a projection in the therapeutic use of mucin as antioxidant must be carefully considered.

GSH, an abundant antioxidant in lung epithelial cells and in ELF [165, 166], is the key to modulate the development of inflammatory-oxidative pulmonary injury. Reduction of H_2O_2 and lipid hydroperoxides is carried out together with GSH-Px or peroxiredoxin. In both cases, GSH is converted in GSSG and quickly reduced by glutathione reductase and NADPH, or it is used in the process of protein folding in the endoplasmic reticulum. In the latter, GSSG is recycled by the protein disulfide isomerase and converted in GSH.

In hypoxia, Jenkinson et al. [167] measured GSSG in isolated perfused rat lungs. Firstly, a period of anoxia (95% N_2 and 5% CO_2) and subsequent reperfusion (95% O_2 and 5% CO_2) were performed. GSSG only was increased during reoxygenation in both perfusate and lung tissue. This suggested the presence of H_2O_2. In another study, Jackson and Veal [168] used a hypoxia and reoxygenation (collapse and reexpansion) model in lungs of rabbits. In this case, GSSG was significantly increased in the alveolar lavage fluid and lung tissue after 2 h of reexpansion subsequent to collapse; besides, a significant decrease of the total glutathione was observed.

On the other hand, a research carried out by White et al. [169] on isolated lungs of rats previously exposed to hypoxia demonstrated that the activity of the glutathione redox cycle was effective on pulmonary cytoprotection after increasing H_2O_2 by hyperoxia. This effect seems to depend on hexose-monophosphate derivation, since lungs previously exposed to hypoxia and that were perfused with oxidase glycogen had increased the reduction equivalents of H_2O_2 when compared to lungs that were preexposed to normoxia. This was reflected in the increasing of GSH/GSSG and NADPH/NADPH$^+$ ratios. Therefore, White et al. [169] proposed the existence of an adaptive process to previous hypoxia with more tolerance towards H_2O_2 by increasing of GSH and NADPH in lung tissue. In ELF, GSH concentrations in humans are at least 100 times greater than those found in plasma (0.5–5 μM) [165, 170, 171]. Low-molecular-weight antioxidants having greater concentrations in upper and lower respiratory tract are ascorbate and urate. GSH is present in ELF only in a significant manner. The latter does not play down the importance of the GSH function in the pulmonary oxidative control, since this is incorporated to one of the main antioxidant enzymatic systems [172]. Jackson et al. [173] found a decrease in mitochondrial GSH and MnSOD and an increase in the concentrations of lactate dehydrogenase enzyme and H_2O_2 in lung cell lines subjected to 1% O_2. As you can see, the pulmonary antioxidant system of GSH depends on the capacity of all its components, both enzymatic and *nonenzymatic*, in and out of the cell.

It is well known the importance of urate as a *nonenzymatic* antioxidant in human respiratory tract [174–177]. Nasal lining fluid has very similar levels as plasma (200–300 μM). Although there is high plasma concentration levels of urate, the theory of urate consumption by the bronchoalveolar or nasal fluid does not seem solved. In a study carried out by Van der Vliet et al. [172] no significant correlation of urate concentrations between plasma and ELF was obtained, which strengthens the presence of local maintenance mechanisms of urate.

Secretion of urate is carried out together with mucin, hence making evident its role in controlling the attacks of oxidant agents in the respiratory tract. Indeed, its main results in the antioxidant defense have been observed in oxidative control to O_3, where urate concentrations in nasal lining fluid significantly decrease after being exposed to this element [175]. With regard to hypoxia, there is no convincing evidence about its effects in the modification of urate concentrations in the respiratory tract. Deaton et al. [178] using an animal model for recurrent bronchial obstruction without inflammation did not find significant modifications in urate concentrations in EBC samples of horses. The lack of evidence makes it difficult to quantify and determine the antioxidant contribution of urate in airways.

Ascorbate (vitamin C) has a double role: sweeping the neutrophil oxidants away and reducing the oxidation of vitamin E [179]. The latter allows returning the antioxidant capacity to the vitamin E. Researchers have described different ascorbate concentrations in plasma and ELF obtained from both nasal region and bronchoalveolar zone [166, 180, 181]. The effects of hypoxia on ascorbate concentrations in lungs are scarce. Deaton et al. [178] observed an inverse correlation between H_2O_2 concentrations in EBC and ascorbate concentrations in ELF from horses affected by inflammatory recurrent airway obstruction.

11. Oxidative Damage in Lungs by Hypoxia

Oxidative stress to lipids is one of the most studied phenomena regarding oxidative stress by hypoxia. In that regard, a 75% increase in TBARs in rats initially exposed to 12% O_2 for 48 h and to 10% for 12.5 d was found in lung homogenates [183]. Kiliç et al. [182] found an increase in TBARs in lung homogenates in newborn rats exposed to 8% O_2 for 3 h. This process was prevented by administering a nitric oxide synthase inhibitor (NG-nitro-L-arginine) and by administering a precursor for its synthesis (L-arginine). Hoshikawa et al. [89] found an increase in lipid peroxidation measured as phosphatidylcholine hydroperoxide in lung homogenates after rats were exposed to 10% O_2 for four days. The administration of both N-acetylcysteine and allopurinol prevented the increase of lipid peroxidation. Minko et al. [4] found an increase in TBARs and conjugated dienes of lung homogenates in rats exposed to 6% O_2 during 120 min. This process was diminished by the application of intratracheal liposomes charged with α-tocopherol. Wilhelm et al. [184] found an increase of aldehydes in lung homogenates after being in a normobaric chamber with 10% O_2 for 5 d. Jankov et al. [91] exposed rats to 13% O_2 for 4, 7, or 14 d, finding an increase in lipid peroxidation measured as 8-isoprostane in lung homogenates. Rats exposed to a moderate altitude (1,500 m) during 8 weeks had increased MDA in their lung homogenates. The administration of polyphenol extract in another experimental group decreased hypoxia-induced lipid peroxidation [185]. After exposition to high altitude simulated in the hypobaric chamber for 5 h at 9,144 m, an MDA increase in lung homogenates of rats was found. This change was avoided in rats receiving dexamethasone or a leaf extract rich in flavonoids [186].

In regard to oxidation to hypoxia-induced protein, Rus et al. [187], subjected rats to hypobaric hypoxia to a 225 mmHg barometric pressure during 30 min and later to reoxygenation from 0 to 5 d, finding an increase in oxidative stress to proteins in lung homogenates measured as the increase of nitrotyrosine. In rats' lung sections, an increase of nitrotyrosine was also found as from the fourth day at 13% of O_2 [91]. In another report, newborn rats were exposed to the hyperoxia protocol with hypoxic events (65% O_2 with 10 hypoxic events of 8% O_2 for 10 min) during four weeks, finding an increase in carbonyl concentration in lung homogenates [188].

Regarding the oxidative damage to nucleic acids, an increase of oxidative damage to the gene promoter for VEGF and to the DNA recognition area for HIF-1α was reported in culture of PAECs and PASMCs exposed to 2% O_2 from 3 to 48 h [189]. In another similar protocol, PAECs cells remained in a hypoxic medium (2% O_2 and 5% CO_2) from 3 to 48 h. Using the endonuclease fingerprinting and ligation-mediated PCR techniques, the injuries to mitochondrial DNA and nuclear DNA were determined. In nuclear DNA the injury to the gene promoter region for VEGF was found [190].

12. Pulmonary Oxidative Damage by Hypoxia Assessed by Noninvasive Methods

Difficulties to obtain lung tissue samples are the reason of the low number of studies carried out in humans. For this reason, most of the information currently available regarding the effects of hypoxia on lung redox state changes is derived from studies carried out on animal models which have been previously reported. In order to find a solution to these limitations, non-invasive methods are arising and are currently being developed to study pulmonary diseases. By using these methodologies, the effects of hypoxia on ROS formation were studied, as well as the effects this condition generates over lung redox state.

In patients having respiratory distress, an H_2O_2 increase in EBC was found [191]. Similar results were found by Sznajder et al. [192] when describing H_2O_2 increase in patients having hypoxemic respiratory failure. Climbers increased its H_2O_2 concentration in EBC after descending a 6,125 m volcano. Another experience was carried out in biathletes that remained training at 2,800 m, and an increase in H_2O_2 concentration was found in the expired air [193]. Patients with OSA showed an H_2O_2 increase [194]. Malakasioti et al.

[195] found an increase of H_2O_2 concentration in EBC in children with the same disease. Previous studies, although in different contexts, share hypoxia as at least one of the main causes, hence making models with limitations. For this reason, studies developed in more controlled conditions, in animals, are complementary, such as the one carried out by Wilhelm et al. [97] where EBC of rats exposed to normobaric hypoxia for 7 d with 10% O_2 was obtained. In this condition, an increase of H_2O_2 concentration was found. A similar result was reported in subsequent studies with the same species and at the same O_2 fraction but under different exposed times: three days [196] and five days [184]. The measurements of H_2O_2 in EBC show various results that guarantee H_2O_2 increase in hypoxia; however, the origin of this marker has not been determined yet, due to many different sources that might be involved (NOX, mitochondria, inflammatory cells, and XO).

Another *prooxidant* widely determined in humans (at a lung level) is exhaled NO (ENO) [197, 198]. With regard to this marker, results are not even, and there are many studies demonstrating that nitric oxide in exhaled breath increases in relation to hypoxia. Hence, Mansoor et al. [199] found ENO increases with no differences in the concentrations of nitrite plus nitrate in EBC in subjects climbing 4,342 m. The administration of L-arginine did not generate modifications in both types of samples. Macinnis et al. [200] found ENO concentration increases after two hours in subjects remaining with 12% O_2 in normobaria. Furthermore, these increases were related to acute mountain sickness incidence, finding that ENO was lower in subjects developing the disease. In adult patients with OSA, an increase in ENO was found and was directly related to the total sleeping time with saturation of hemoglobin by O_2 below 90%.

There are other results indicating that ENO decreases or does not modify by hypoxia. In subjects breathing 12% O_2, ENO decrease was found after two hours in subjects susceptible to developing high altitude pulmonary edema. Moreover, the percentage change of ENO was correlated inversely with pulmonary artery pressure after 2 h [201]. Under hypobaric hypoxia conditions, Hemmingsson et al. [202] found that subjects had decreased ENO when exposed acutely to 5,000 m in a hypobaric chamber, meanwhile this phenomenon was not observed when carried out in a normobaric hypoxia laboratory (11.3% O_2). Donelly et al. [203] carried out ENO measurements in humans until reaching saturation of hemoglobin for 80% O_2 in the laboratory and then in a climb to 5,050 m in land conditions finding that ENO decreased in hypobaric hypoxia, meanwhile it remained the same in normobaric. In both situations, pulmonary artery pressure increased; therefore, authors came to the conclusion that ENO probably does not participate in tone regulation of pulmonary artery in hypoxia. In longer permanence in hypoxia as the one carried out by Güzel et al. [204] at 2,300 m for seven days, a decrease in ENO was also found. Miners staying sporadically (3 weeks in altitude versus 3 weeks at 800 m) for more than one year in altitude (between 3,600 m and 4,000 m) were measured at 4,000 m, finding a decrease in ENO. Children exposed chronically to altitude did not show differences in ENO—children from Aymara ethnic group versus children having European descent, although the first ones had lower pulmonary artery pressure. The diversity of the results, increased, decreased, or remaining levels of NO, shall be discussed as for the meaning of this parameter in different scenarios where hypoxia participates. All causes of different results are also not known, nevertheless, to a considerable extent, decreases have been attributed to the lack of O_2 (substratum to form NO). Consequently, Schmetterer et al. [20] found that 10 min administration of O_2 from 10% to 100% in humans, oxygen pressure, and ENO were directly related. Another cause is related to the type of hypoxia, hence, the results of hypobaric are not alike to normobaric [202, 203]. Finally, measurements of ENO have variations attributed to the method and equipment, requiring the modification of measurement methods, as it was suggested by Hemmingsson et al. [202].

Oxidative stress in lungs by hypoxia has also been measured in EBC samples; hence, Araneda et al. [205] found MDA increases measured in EBC after 10 d starting from 3,000 m in subjects climbing 6,125 m. In the same work, MDA concentration increases were found in the same type of samples in subjects that carried out a maximal cycloergometer exercise at 2,160 m altitude, meanwhile this same physical effort did not generate changes at 670 m. In a subsequent study, 8-isoprostane was determined in exhaled breath samples in biathletes that trained at 2,800 m for six weeks, finding an increase tendency of this marker [193]. Patients having OSA showed an 8-isoprostane concentration increase in EBC after using oxygen with continuous positive airway pressure. This parameter returned to normal values [194]. Similar results were found by Carpagnano et al. [206], also in patients with this same disease.

13. Conclusions

Early studies on hypoxia have reported the decrease of ROS. Nowadays, with the development of methodologies based on oxidation-sensitive proteins, it is known that ROS and oxidative damage increase in hypoxia. In lungs, ROS sources by hypoxia are multiple: in the mitochondrial ETC they are mainly formed in the complexes I and III, which increases ROS in the intermembrane space and cell cytoplasm. The enzyme XO/XD has a proven role as ROS generator by hypoxia in lungs, being, probably, one of the most important ROS sources. Another source is NOx, which has a large representation in lungs. Current records point out that, from the various isoforms, the ROS formation from this system is mainly originated in NOX4, this isoform being also associated with vascular remodeling and proliferation. From the three NOS isoforms, the inducible variety, activated in this context by NF-κB, HIF-1α, and ROS, has a clearer role in the ROS formation under hypoxic conditions. The inflammatory process, which is developed by hypoxia, provides alveolar macrophages that secrete proinflammatory substances and ROS, which promote the infiltration of PMNL, ROS secretor activators.

In regard to antioxidant systems, from all the works that have studied this compound, it is possible to conclude that there is great variety of results. SOD is the most studied antioxidant, showing modifications of mRNA, amounts and activity in both mitochondrial and extracellular varieties. Studies on animals overexpressing Ec-SOD are probably the ones that make the role of this isoform in hypoxia clearer for being a protector of the development of pulmonary hypertension by chronic hypoxia. Catalase and glutathione peroxidase have demonstrated a great variety of results, from nonshowing any variation to increases and decreases; although, in cultured cells transfected with these enzymes, a decrease in the formation of ROS by hypoxia has been observed.

In relation to TRx, available results do not allow directly knowing their modifications by hypoxia. We do know that their administration as a recombinant protein has a protector effect against ischemia and reperfusion of this organ. Regarding nonenzymatic antioxidants against hypoxia, there is almost exclusively information about glutathione and the decrease of the relation GSH/GSGG against hypoxia; besides, on the basis of its concentration, its protector role as part of the ELF is speculated.

It is a fact that the great difficulty for studying oxidative damage by hypoxia in lungs, in all species including humans, is because of the difficulties to obtain tissue, the current evidence being mostly obtained from studies conducted whether in cells and pieces of organs or isolated complete organs. In this sense, it is of utmost importance to check and transfer many of the results presented herein to whole bodies and humans, too. In order to accomplish this task, it is necessary to move forward in using and developing methodologies, minimally invasive for the study of this phenomenon, as well as developing new ways that allow direct studies on humans.

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Review Article

Kluyveromyces lactis: A Suitable Yeast Model to Study Cellular Defense Mechanisms against Hypoxia-Induced Oxidative Stress

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Studies about hypoxia-induced oxidative stress in human health disorders take advantage from the use of unicellular eukaryote models. A widely extended model is the fermentative yeast *Saccharomyces cerevisiae*. In this paper, we describe an overview of the molecular mechanisms induced by a decrease in oxygen availability and their interrelationship with the oxidative stress response in yeast. We focus on the differential characteristics between *S. cerevisiae* and the respiratory yeast *Kluyveromyces lactis*, a complementary emerging model, in reference to multicellular eukaryotes.

1. Introduction

Interest in hypoxic and oxidative stress studies is increasing in recent years, mostly in relation to aging or diseases such as neurodegenerative disorders or cancer. These processes in human cell lines show a very complex regulation, and therefore the availability of simple models is extremely useful. Yeasts have demonstrated to be suitable unicellular eukaryotic models since, in addition to generalized culture facilities, global "omic" analysis is fully developed and molecular mechanisms are generally well conserved. For instance, although obviously without nervous system, most of the molecular signaling pathways and the proteins involved in human neurological diseases are functionally conserved in yeasts [1]. Besides, functional characterization of human genes is sometimes achieved by means of their heterologous expression in mutant yeasts.

Most studies about the hypoxic and oxidative stress responses and their connections have been carried out hitherto on *Saccharomyces cerevisiae*, a yeast model with a predominantly fermentative metabolism [2]. In the same way, *S. cerevisiae* mutants have been frequently used as research models in aging [3] and in human pathologies [4]. Frequently, the mechanisms discovered with this yeast proved to be conserved in multicellular eukaryotes. However, human tissues such as the neuronal network have oxidative metabolism, and therefore the use of alternative yeast models has been suggested [5]. We analyze *Kluyveromyces lactis* from the perspective of an alternative eukaryote model in these studies or similar studies, since this yeast has a predominantly respiratory metabolism.

Molecular mechanisms that support the metabolic differences between *S. cerevisiae* and *K. lactis* and the specific responses to hypoxia or oxidative stress have been studied. Redox metabolism is a key differential point between *S. cerevisiae* and *K. lactis*, both thiol-redox and NAD(P)H-redox reactions. *K. lactis* is characterized, opposite to *S. cerevisiae*, by a higher glucose flow through the pentose phosphate pathway (PPP) than through glycolysis [6] and as a consequence by a higher production of NADPH in the cytosol. In fact, one of the molecular keys supporting the difference in the Crabtree phenotype (inhibition of respiration by fermentation) of the two yeast species lies in the mechanisms involved in the re-oxidation of the NADPH [7, 8]. A significant part of this reoxidation is carried out in *K. lactis* by mitochondrial external alternative dehydrogenases (NDEs), which use NADPH, the enzymes of *S. cerevisiae* being NADH-specific. Unlike *S. cerevisiae*, transcription of NDEs genes in *K. lactis* is not regulated by the carbon source. Since NDEs may compete with alcohol dehydrogenases for the cytosolic NADH, their repression at high glucose concentrations, as it occurs in *S. cerevisiae*,

correlates with an increase of reoxidation of glycolytic NADH by the alcohol dehydrogenases and therefore with the prevalence of aerobic fermentation and the Crabtree-positive phenotype [9]. Interestingly, NDEs have been reported to influence ROS production and life span in *S. cerevisiae* [10, 11]. Moreover, the NADPH-dependent pathways of response to oxidative stress also contribute to NADPH reoxidation. In *S. cerevisiae*, they play a main role together with glutamate dehydrogenase and also operate, although to a lesser extent, in *K. lactis*, [8, 12, 13].

In this paper, we focus on the complex interdependence of multiple mechanisms, which arise as a consequence of the decrease of oxygen availability and on the responses elicited to compensate this stress. A general overview of all the subjects analyzed is shown in Figure 1. Along the text, special reference is made on the differences found between *S. cerevisiae* and *K. lactis*, looking for the potential advantages and disadvantages of these models in reference to each other and in comparison to multicellular eukaryotes.

The first intracellular signal sensing low levels of oxygen is the heme content. The biosynthesis of heme includes enzymes that directly use oxygen as electron acceptor during the catalysis, and, besides, several steps are regulated by oxygen availability. Other important pathway that directly uses oxygen is the biosynthesis of ergosterol, which is also regulated by oxygen availability. The intake of ergosterol from the media through the membrane is also regulated by oxygen levels. Downstream in these sensing strategies is heme and ergosterol dependent transcriptional factors, which act in the nucleus to regulate the transcription of more than 100 genes, those conditioning the "hypoxic response" and improving the use of the low levels of oxygen. Hypoxia signals the activation of mechanisms that regulate the transcription of genes involved in the oxidative stress response. Simultaneously, the decrease in oxygen levels causes a complete rerouting of nutrients through different metabolic pathways. This principally affects glucose and other sugars, which can follow fermentative or respiratory transformations and, in turn, condition the systems of redox exchange between cytoplasm and mitochondria and the mechanisms that produce ROS. Reoxidation of reduced NAD(P)H also has regulatory effects on the diverse metabolic routes that need the oxidized coenzyme forms to function. ROS also elicit other mechanisms of cell defense, including reoxidation of NAD(P)H and life span adjustment, programmed cell death, and mitophagy (Figure 1).

2. The Hypoxic Response in *K. lactis*

During hypoxia, it is advantageous for cells to adapt the pattern of gene expression in order to improve oxygen utilization. The hypoxic response is well documented in the model yeast *Saccharomyces cerevisiae,* whose cells sense oxygen via the levels of heme and sterols [14–17]. The response of *S. cerevisiae* to hypoxia produces increased expression of genes related to ergosterol synthesis, cell wall composition, and glycolytic genes and reduced expression of components of the respiratory chain, ATP synthesis, and the citric acid cycle [14, 18, 19]. However, this knowledge is not directly applicable to other yeasts, even to those close-related in phylogeny, which became evident in the last years with the advances in the study of the hypoxic response in *K. lactis* and other yeasts. It has been proposed that a whole-genome duplication (WGD) contributed to yeast evolution from strict aerobes to facultatives/fermentatives [20–22]. Functional specialization between duplicated genes explains the existence in *S. cerevisiae* of homologous genes (COX5a/COX5b; CYC1/CYC7; HYP2/ANB1; AAC1/AAC2/AAC3) differentially expressed in aerobic and hypoxic conditions [14, 23–25]. *Kluyveromyces lactis* does not present duplication of genes with specialized aerobic, hypoxic transcription, but the unique copy is regulated by oxygen availability [26]. Probably, *K. lactis* and *S. cerevisiae* are diverged from one common ancestor yeast previously to the WGD event and this could explain the multiple differences observed when comparing the response to hypoxia in both yeasts, as explained below.

Although *K. lactis* is unable to grow under strictly anoxic conditions [27], probably due to the absence of expression of genes related to the import of sterols in this condition [28], this yeast ferments sugars and grows in hypoxic conditions defined as oxygen availability below 1% of fully aerobic levels [7, 29]. Several reports of genes upregulated during hypoxia in *K. lactis* have been published. A *K. lactis* heme-deficient strain, obtained by *KlHEM1* disruption, was used to assess the functional significance of heme-directed regulation in *K. lactis*; *KlHEM13*, encoding the coproporphyrinogen oxidase (EC 1.3.3.3), an oxygen-requiring enzyme that catalyzes the sixth step of heme biosynthesis, was the first hypoxic gene functionally characterized in this yeast [30, 31]. Other gene from the heme biosynthetic pathway, *KlHEM1*, is upregulated during hypoxia in *K. lactis* [32]. The *KlPDC1* gene, encoding for the enzyme pyruvate decarboxylase, is also induced by hypoxia [33]. After the completion of the Génolevures sequencing project [34], the availability of the complete sequence of the *K. lactis* genome allowed the design of specific DNA arrays containing selected DNA probes putatively related to the aerobic-hypoxic response by their similarity to the orthologs in *S. cerevisiae* [26]. The nature of the hypoxic transcriptional response in *K. lactis*, as revealed by using these arrays, differed notably from *S. cerevisiae*, but confirmed the existence of hypoxic upregulated genes in *K. lactis* such as *KlOYE2 (KLLA0A09075g)*, *KlGSH1* (*KLLA0F14058g*) and *KlOLE1* [26]. Besides *KlOLE1,* the transcription of other lipid biosynthetic genes like *KlERG1*, *KlFAS1*, *and KlATF1* is also induced by hypoxia [35].

In *S. cerevisiae*, adaptation to hypoxia requires the transcriptional induction or derepression of multiple genes organized in regulons controlled by specific transcriptional regulators. Considering that in *K. lactis* the hypoxic response exists but the target genes are not coincident and are not equally regulated, the question arose about the functionality of sensors and transcriptional regulators. The principal sensors in the yeast response to hypoxia are heme and ergosterol. The heme biosynthetic pathway is well conserved in different organisms throughout evolution [36], and this is also true between *S. cerevisiae* and *K. lactis.* Both yeasts have eight highly homologous genes necessary for the biosynthesis of

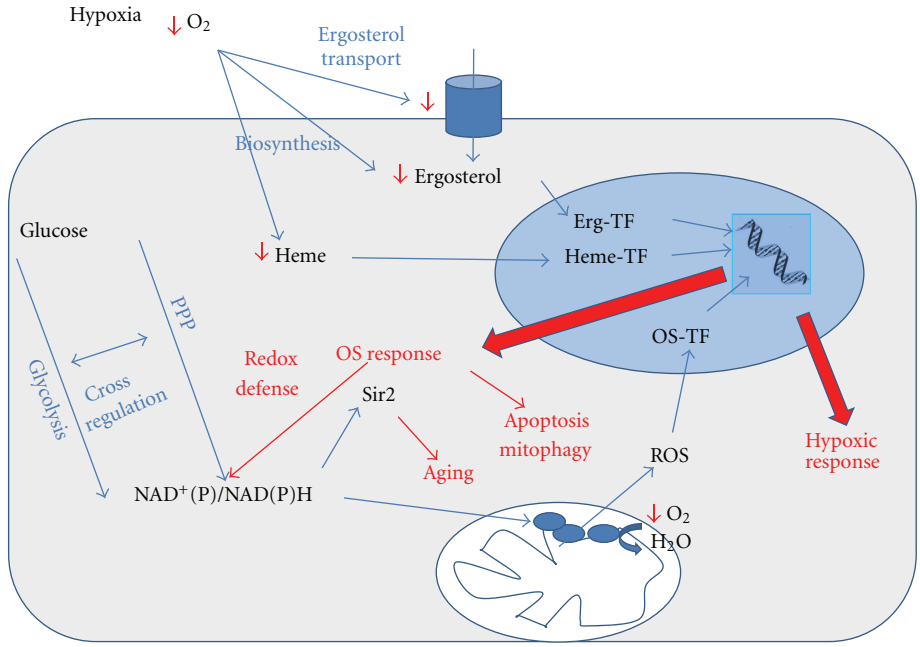

Figure 1: A panorama of the multiple connections between hypoxia, metabolic rerouting, oxidative stress response, and cell defense mechanisms. Erg: ergosterol; PPP: pentoses phosphate pathway; TF: transcriptional factors.

heme. For the three genes of the heme biosynthetic pathway characterized in *K. lactis* to date (*KlHEM1*, *KlHEM12*,and *KlHEM13)*, functional equivalence with their *S. cerevisiae* homologs has been confirmed experimentally by cross-complementation [31, 32, 37]. As it happens in *S. cerevisiae*, the transcriptional regulation of *KlHEM12* is not a key point for regulation of heme synthesis in *K. lactis* [38] and its transcriptional regulation in different carbon sources [38] is also similar to that reported for its homolog in *S. cerevisiae* [39, 40]. However, notable differences exist in the regulation of the other two characterized genes. In *S. cerevisiae*, the expression of *HEM1* is constitutive [41], but in *K. lactis* the first step of the heme biosynthesis is under double-feedback regulation by heme, at the level of gene transcription [37] and mitochondrial import of the protein [42]. Although in *S. cerevisiae* the aerobic repression of *HEM13* is mediated by the transcriptional regulator Rox1p, diverse data indicate that the mechanism is different in *K. lactis* and a similar repressor does not operate [43, 44].

In *S. cerevisiae*, intracellular levels of heme regulate the activity of the transcriptional regulator Hap1 [14]. The CCAAT-binding complex Hap2/3/4/5, an evolutionarily conserved multimeric transcriptional activator in eukaryotes [45], is also necessary for the activation of many genes involved in respiratory metabolism [45], although its direct activation by heme has not been demonstrated. Targets of Hap1 include genes required for respiration and for controlling oxidative damage [46–48] and also the aerobic repressor Rox1. When the oxygen levels drop, heme does not bind to Hap1. Then, the interaction with Ssa1, Ydj1, and Sro9 maintains to Hap1 inactive [49, 50]. As a result, *ROX1* is not expressed and no longer represses aerobic expression of genes involved in the hypoxic response. Moreover, Mot3 collaborates in the repression exerted by Rox1 in target promoters [51], and Ixr1 has been related to the hypoxic response of *S. cerevisiae* in cross-regulation with *ROX1* [52–54]. Although several homologs to the components of the Hap2/3/4/5 complex have been cloned in *K. lactis* [55–57], the respiratory system of *K. lactis,* escapes from *HAP2* control [56]. Contrary to data previously described for the homologous gene of *S. cerevisiae*, the function of the *KlHAP1* gene does not affect growth in media with carbon sources used by fermentative or respiratory pathways in *K. lactis* and *Kl*Hap1 is not a transcriptional activator of the expression of genes related to respiration or sterol biosynthesis [58] but represses the expression of the major glucose transporter [59]. In a similar way, *KlROX1* does not regulate the hypoxic response in *K. lactis* [60] and the *KlROX1* promoter is not regulated by *Kl*Hap1 or *Kl*Rox1 in response to changes aerobiosis/hypoxia [44].

Parallel, Hap1-Rox1-independent, oxygen response pathways exist in yeast. For instance, in *S. cerevisiae*, the transcription of the hypoxic gene *OLE1* depends on cytochrome *c* oxidase [61] and requires the transcription factor Mga2 that is functional in hypoxia [62]. In *K. lactis,* this regulatory circuit is also different and, although *KlMGA2* shows homology to the *MGA2* gene from *S. cerevisiae, Kl*Mga2does not regulate *KlOLE1* hypoxic expression [35]. Sut1 and Sut2 are also involved in the transcriptional induction of hypoxic genes and in sterol uptake and synthesis in *S. cerevisiae* [63, 64].

Another sensing pathway includes the regulators of sterol biosynthesis Upc2 and Ecm22 [65]. Sterol depletion leads to activation of the paralogous genes *UPC2* and *ECM22* [16], which control expression of a subset of hypoxic genes. Both bind to a sequence motif known as the sterol

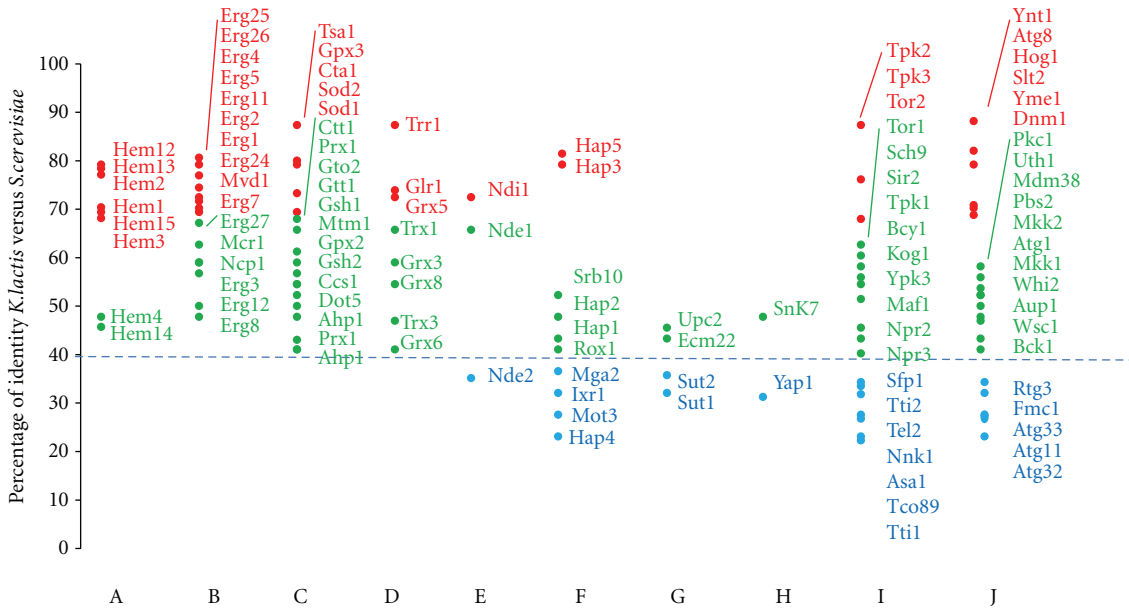

Figure 2: Homologies (percentage of identity calculated according to BLASTp in Génolevures) between *K. lactis* and *S. cerevisiae* proteins related to the pathways summarized in Figure 1. Red, 100–70% identity; green 69–40% identity; blue, <40% identity. (A) heme biosynthesis; (B) ergosterol biosynthesis and supply; (C) NAD(P)H consuming oxidative defense reactions; (D) other oxidative defense reactions; E, NAD(P)-dehydrogenases from the inner membrane of mitochondria; (F) heme/respiration-related transcriptional factors; (G) sterol-related transcriptional factors; (H) peroxide-related transcriptional factors; (I) life span-related proteins; (J) mitophagy-related proteins.

regulatory element (SRE) in the promoters of their target genes, but Ecm22 is an aerobic repressor and Upc2 an anaerobic activator, which is upexpressed during hypoxia. They regulate expression of ergosterol biosynthesis genes and the DAN/TIR family of cell wall proteins [65–67]. This regulatory circuit remains unstudied in *K. lactis,* although analysis of the genome sequence shows that the hypoxic genes from the sterol biosynthetic pathway are conserved in the two yeasts [26].

Quantitation of the homology between proteins translated from verified or putative orthologous genes of *K. lactis* and *S. cerevisiae* reveals that, with few exceptions, the proteins related to biosynthetic routes producing intracellular changes in heme and ergosterol are more conserved than the transcriptional factors, which are regulated by their levels and produce the hypoxic response (Figure 2).

3. Oxidative Stress Response in *K. lactis*

The oxidative stress response in *K. lactis* is a mostly unexplored field. The number of published works is less than 1.5% compared to *S. cerevisiae*. Several studies based on comparative genomics have been performed, combining *in silico* and experimental approaches [26, 68, 69]. The search in the *K. lactis* genome of putative *S. cerevisiae* orthologs related to the oxidative stress response (genes coding for superoxide dismutases and their chaperones, catalases and peroxidases, proteins of the glutathione, and thioredoxin systems) has suggested that pathways and proteins are generally conserved, with a few exceptions mainly affecting gene redundancy or predicted subcellular location of the proteins [69]. These exceptions comprise the groups of genes encoding thioredoxin peroxidases, thioredoxin reductases, thioredoxins, glutaredoxins, and glutathione transferases (Figure 2). However, several functional differences affecting mainly connections between redox pathways related to carbohydrate metabolism, respiratory functions and oxidative stress response are observed [69]. In support of these results, a recent study about glutathione transferases, and synthetases in yeast species representatives of fermentative, respiratory, and oxidative metabolism [70] found significant differences in homology and predicted intracellular sorting.

Among the reported differences, it is remarkable to outline the role of the thiol-redox pathways, specifically glutathione reductase (GLR), the enzyme that catalyzes the interconversion of oxidized (GSSG) and reduced glutathione (GSH) using NADPH as reducing power. Whereas in *S. cerevisiae* the expression of GLR increases in response to oxidative stress produced after addition of peroxides by a Yap1-mediated mechanism, this effect is absent in *K. lactis* [26, 71, 72]. Surprisingly, both *S. cerevisiae* and *K. lactis* GLR depletion mutants are more sensitive to oxidative stress [12, 13]. In such *K. lactis* GLR mutants, increase in ROS production, catalase, and thioredoxin reductase (TRR) activities are observed and the expression of a pool of other antioxidant enzymes and oxidoreductases is also upregulated [73]. It is likely that TRR and other NADPH-dependent oxidoreductases might replace GLR in maintaining the GSH/GSSG ratio. In fact, purified *K. lactis* TRR shows GLR activity *in vitro* (our unpublished results). In support of this explanation, it has been reported that the thioredoxin-TRR system can reduce GSSG in *S. cerevisiae* [74]. Other reported functional differences affecting the OS defense enzymes of *K. lactis* and *S. cerevisiae* are regarding the mechanism of

cation handling of the superoxide dismutase Sod1 [75] and the transcriptional regulation of the *SOD1* gene after a shift to hypoxia [26, 76]. Also the transcriptional regulation of the genes encoding catalases [12, 26, 77, 78] and glutathione synthetases [26, 79] under aerobic/hypoxic conditions and under peroxide-treatment is different in the two yeasts. About comparative analyses between the transcriptional factors related to the oxidative stress response in *S. cerevisiae* and *K. lactis*, Yap1 and Snk7 share, respectively, 33% and 50% identities (Figure 2), and *Kl*Yap1 has been functionally characterized in relation to the oxidative stress response induced by metals and peroxides [80].

Several evidences support that in *K. lactis* the OS response has a regulatory role upon fermentation/respiration balance. Thus, there is a positive correlation between the increase of GLR activity and the glucose-6-phosphate-dehydrogenase activity (from PPP) when oxygen levels increase [12]. Besides, the glucose respiration rate, in *K. lactis* cells that metabolize all the monosaccharide through the PPP, increases upon GLR depletion and decreases upon GLR overexpression [13]. Proteome analysis reveals that there is a different response to H_2O_2-treatment, which is dependent on GLR in such a way that the expression of several enzymes of the glycolysis and the Krebs' cycle decreases in the wild-type strain, while enzymes of these pathways and the PPP increase in the GLR depleted mutant [73]. Other indirect evidence is that *K. lactis* GLR activity decreases in a Gcr1-mutant [13] being Gcr1 at the same time a positive transcriptional regulator of *KlNDI1* [9]; *KlNDI1* is the gene encoding the internal mitochondrial alternative dehydrogenase (NDI), the enzyme that replaces the respiratory chain complex I found in other eukaryotes [81]. GLR depletion mutants grow better in glucose than the wild type, overall when all the glucose is metabolized through the PPP, which might be explained by rerouting the oxidation of the NADPH produced in the PPP from GLR to NDEs, thus increasing ATP production in the respiratory chain [12, 13].

Yeasts alternative dehydrogenases of the mitochondrial inner membrane are also related to OS and are other point of connection with the response to metabolic changes produced by oxygen availability. The transcription of the two *K. lactis* genes encoding NDEs decreases when cells are under oxidative stress and the NADPH-related defense mechanisms are activated [9, 82]. Regarding *K. lactis* NDI, we have proved that the transcription of the *KlNDI1* gene is induced in nonfermentable carbon sources through a process mediated by the factor Adr1 and that the expression of the gene did not decrease after an hypoxic shift [9]. The homologous *S. cerevisiae* enzyme has been more widely studied and its role in aging and ROS production has been reported [83]. Differences found in yeasts NDI are of clinical interest since they have been used in gene therapy of diseases [84] such as Parkinson's disease [85] or hereditary optic neuropathy [86, 87]. Heterologous expression of the *S. cerevisiae NDI1* gene reduces the overproduction of ROS caused by mitochondrial complex I defects in multicellular eukaryotes [88].

These connections between thiol-redox OS reactions and carbohydrate metabolism described above in *K. lactis* are also supported in other organisms. Ralser et al. [89] discovered that *S. cerevisiae* cells with reduced activity of the key glycolytic enzyme triose-phosphate isomerase exhibit an increased resistance to the thiol-oxidizing reagent diamide. This phenotype is conserved in *Caenorhabditis elegans* and the underlying mechanism is based on a redirection of the metabolic flux from glycolysis to the PPP, altering the redox equilibrium of the cytoplasmic NADP(H) pool. Another key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is known to be inactivated in response to various oxidant treatments, and this causes a similar redirection of the metabolic flux [89].

4. The Hypoxic-Induced Oxidative Stress Response in *K. lactis*

A connection between the hypoxic and oxidative stress responses has been reported in the fermentative yeast *S. cerevisiae*. Several *S. cerevisiae* genes that are induced during hypoxia are related to the oxidative stress response. *CUP1* and *CUP2*, which are necessary for the removal of superoxide radicals, are upregulated 11.6-fold during hypoxia in a Rox1 and Srb10-dependent mechanism [18]. Other genes related to oxidative stress (*HSP12*, *FMP46*, and *GRE1*) DNA repair (*ALK1*) or mitochondrial genome maintenance (*MGM1*)also increase their expression during hypoxia [18]. The level of mitochondrial and cytosolic protein carbonylation, the level of mitochondrial and nuclear DNA damage measured by 8-OH-dG modification, and the expression of *SOD1*, encoding superoxide dismutase, increases transiently during a shift to anoxia [76]. Besides, the specific proteins, which become carbonylated during the shift to anoxia, are the same proteins that become carbonylated during peroxidative stress. These results demonstrate that yeast cells exposed to anoxia experience transient oxidative stress and suggest that ROS generated could also signal the variation in oxygen levels and trigger the nuclear response to hypoxia affecting transcription [76]. However, the specific connection between ROS production, protein, or DNA modifications and transcriptional regulation has not yet been elucidated in yeasts. The question about whether mitochondrial or cytosolic proteins, which are specifically oxidized in cells exposed to anoxia, play a role in signaling pathways from the mitochondrion to the nucleus that function to induce hypoxic genes remains unanswered.

In *K. lactis,* after analyzing 30 genes related to oxidative stress, only two (*KlGSH1* and *KlOYE2*) increased their expression after the hypoxic shift [26]. However, a whole-genome approach has not been carried out in this yeast and, therefore, a direct comparison of these data with those reported from *S. cerevisiae* is not accurate. An interesting observation, which suggests that also in *K. lactis* the hypoxic response might be triggered by ROS production, is that the hypoxic response is highly dependent on the relative flux of glucose through glycolysis or the pentose phosphate pathway (PPP). The predominant use of PPP *versus* glycolysis is accompanied by a higher expression of mitochondrial cytochrome *c* [7], which might be associated with the mitochondrial chain activation and changes in ROS production.

Indeed, in a *rag2* mutant, lacking phosphoglucose isomerase and committed to reroute the glucose-6-phosphate through PPP, in order to bypass the blocked glycolytic step, a more intense hypoxic response than the wild-type strain, and that affects the genes of heme metabolism and the oxidative stress response, is observed [26].

A new link between oxidative stress and hypoxia comes from the analysis of multiple functions attributed to transcriptional regulators initially characterized in the aerobic/hypoxic response. In *S. cerevisiae,* Hap1 not only acts as an aerobic activator but has a regulatory function during hypoxia [17, 90]. Hap1 also controls the expression of genes related to sterol biosynthesis [14, 91–94] and *SOD2,* involved in the oxidative stress response [95].

Although putative homologues of two of the principal regulators of the aerobic/hypoxic response in *S. cerevisiae* (Hap1 and Rox1) have been characterized in *K. lactis*, their sequence and function diverge notably from those described in *S. cerevisiae* [44, 58]. Remarkably, their functions in *K. lactis* are somehow related to the metal-induced oxidative-stress response. Deletion of *KlHAP1* increases the resistance to oxidative stress or cadmium [58]. Moreover, the induction by 0.5 mM H_2O_2 of two genes related to the oxidative stress response, *KlYAP1* and *KlTSA1,* is repressed by *Kl*Hap1p [58]. *KlROX1* mediates the response to arsenate and cadmium [44]. KlRox1p binds to the *KlYCF1* promoter, a gene related to cadmium detoxification, and causes its activation [44].

5. Adjustment of Cell Survival and the Oxidative Stress Response

Besides the transcriptional, proteomic, and metabolomic reorganization caused by the oxidative stress, ROS also elicit other mechanisms of cell defense, including life span adjustment, programmed cell dead, autophagy, and mitophagy. Mitochondria, being the major intracellular source of ROS, are involved in aging and life span regulation [96] Yeasts have been proved to be good models for studying these processes. In *S. cerevisiae,* a cross-regulation between glycolysis and PPP has been proposed in order to prevent oxidative stress when cells switch from anaerobic to oxidative metabolism. Low activity of the glycolytic enzyme pyruvate kinase causes accumulation of PEP and blocks the pathway diverting the glucose flux into the PPP [97]. This mechanism helps to balance the increased ROS production during oxidative metabolism [97]. Also in mammals, during oxidative damage in cancer cells, a similar redirection of metabolic fluxes contributes to ROS clearance [98]. Therefore, it is possible that the hypoxic and the oxidative stress responses, influenced by the reorganization of the utilization of different metabolic pathways, also contribute to modulate these cell defense mechanisms in yeasts and other cells.

Studies pioneered in *S. cerevisiae* by measuring life span have revealed several molecular mechanisms underlying cellular aging and which are well conserved in eukaryotes. Two basic experimental approaches have been applied in unicellular organisms. Replicative life span (RLS) is defined as the number of daughters a single cell produces during its life [99]. Chronological life span (CLS) is defined as the time a population of cells survive in stationary phase [100]. In *S. cerevisiae*, a Crabtree-positive yeast, calorie restriction by glucose limitation increases both RLS and CLS, a feature that coincides with increased cytochrome content, and NADH-cytochrome *c* reductase activity [101]. In. *K. lactis*, a Crabtree-negative yeast for which glucose limitation does not promote an enhancement of the respiratory capacity [2], the increase in CLS by glucose limitation is not produced [102]. These results suggest that calorie restriction-dependent increase in longevity may be due to mitochondrial control and more particularly the regulation of oxidative phosphorylation activity.

An additional nexus between aging and the redox cell balance came from the discovery of sirtuins. They are NAD^+-dependent enzymes and they belong to a highly conserved family of proteins that in yeasts, invertebrates, and mammals act in diverse functions related to longevity [103]. In *S. cerevisiae,* Sir2 is induced in cells treated with 4 mM H_2O_2 or 10 mM menadione [104] and these data suggest a connection between oxidative stress, Sir2 activation and longevity. The existence of complexes of Sir2 with other metabolic enzymes NAD^+-dependent, like those formed with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase Tdh3 or the alcohol dehydrogenase Adh1 [105], might indicate that the ratio NAD^+/NADPH in the microenvironment surrounding Sir2 could act as its modulator. Recently, a third complex of Sir2 with Mdh1, the mitochondrial malate dehydrogenase, has been proposed based on multiple common network interactions involving also the proteins Rad53, Aat1, Fob1, and Hst1 [106], although not yet proved by physical interactions. Since Mdh1 is overexpressed under conditions of calorie restriction [107] and it causes extension of Sir2-dependent RLS [108], further investigation is promising. In *K. lactis* sirtuins or its regulators have not been studied but there is an ORF (KLLA0F14663g) with 62% identities to *S. cerevisiae* Sir2 (Figure 2). Considering the importance of sirtuins and their modulators in the etiology and treatment of human pathologies such as metabolic, cardiovascular, and neurodegenerative diseases or cancer [104, 109] and the similarities found between the high respiratory metabolism of *K. lactis* and certain human cell types like neurons [5], sirtuins and related proteins from *K. lactis* are good targets for study.

About the signaling pathways that affect life span in yeast (reviewed in [103]), the serine threonine kinases Tor1, Sch9, and PKA that control nutrient signaling pathways also regulate aging in yeasts, and their homologs in animals share conserved functions in aging. Depletion of Tor1 kinase increases both RLS and CLS in budding yeast, flies, and *C. elegans*. Likewise, PKA kinase activation lengthens life span in budding yeast and longevity in mice. The kinase S6K1, which is known to be related to the control of aging in mice, Drosophila, *C. elegans,* and the yeast Sch9 kinase that controls RLS and CLS in yeast represent other group or orthologous genes. Purification of TOR from yeast and human cells revealed that TOR can exist in at least two multiprotein complexes, termed TORC1 and TORC2 [110]. Interestingly, it has been found that superoxide anions

Table 1: Putative main actors of aging and its signaling pathways in *K. lactis* and *S. cerevisiae*. Degree of identity (%) between homologs is indicated in Figure 2.

Protein	ORF *K. lactis*	ORF *S. cerevisiae*	Function
Sir2	YDL042C	KLLA0F14663g	NAD^+ -dependent histone deacetylase
Tpk1	YJL164C	KLLA0B12716g	PKA catalytic subunit
Tpk2	YPL203W	KLLA0D03190g	PKA catalytic subunit
Tpk3	YKL166C	KLLA0B07205g	PKA catalytic subunit
Bcy1	YIL033C	KLLA0E04181g	PKA regulatory subunit
Ypk3	YBR028C	KLLA0F24618g	An AGC kinase phosphorylated by cAMP-dependent protein kinase (PKA) in a TORC1-dependent manner
Asa1	YPR085C	KLLA0D09086g	Subunit of the ASTRA complex involved in the stability or biogenesis of PIKK*s such as TORC1
Tor1	YJR066W	KLLA0B13948g	PIK-related protein kinase and rapamycin target, subunit of TORC1, a complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport, and autophagy, involved in meiosis
Tor2	YKL203C	KLLA0B13948g	PIK-related protein kinase and rapamycin target, subunit of TORC1 and TORC2, a complex that regulates cell-cycle dependent polarization of the actin cytoskeleton, involved in meiosis
Nnk1	YKL171W	KLLA0A06776g	Protein kinase, implicated in proteasome function, interacts with TORC1, Ure2, and Gdh2
Tco89	YPL180W	KLLA0E18855g	Subunit of TORC1 (Tor1 or Tor2-Kog1-Lst8-Tco89)
Kog1	YHR186C	KLLA0A09471g	Subunit of TORC1, it may act as a scaffold protein to couple TOR and its effectors
Tti1	YKL033W	KLLA0F25762g	Subunit of the ASTRA complex, involved in chromatin remodeling, telomere length regulator involved in the stability or biogenesis of PIKK*s such as TORC1
Tti2	YJR136C	KLLA0B04026g	Subunit of the ASTRA complex, involved in chromatin remodeling, telomere length regulator involved in the stability or biogenesis of PIKK*s such as TORC1
Sch9	YHR205W	KLLA0B03586g	AGC family protein kinase and functional ortholog of mammalian S6 kinase, phosphorylated by Tor1p and required for TORC1-mediated regulation of ribosome biogenesis, translation initiation, and entry into G0 phase, integrates nutrient signals and stress signals from sphingolipids to regulate life span
Maf1	YDR005C	KLLA0E17535g	Negative regulator of RNA polymerase III, binds to the N-terminal domain of the Rpc160 subunit of Pol III to prevent closed-complex formation, localization and activity are regulated by phosphorylation, mediated by TORC1, protein kinase A, and Sch9
Tel2	YGR099W	KLLA0D15158g	Subunit of the ASTRA complex, involved in the stability or biogenesis of PIKK*s such as TORC1
Sfp1	YLR403W	KLLA0B03047g	Regulates transcription of ribosomal protein, response to nutrients and stress, G2/M transitions during mitotic cell cycle, and DNA-damage response and modulates cell size, regulated by TORC1 and Mrs6 prion
Npr2	YEL062W	KLLA0D01067g	Npr2/3 complex mediates downregulation of TORC1 activity upon amino acid limitation
Npr3	YHL023C	KLLA0F18238g	Npr2/3 complex mediates downregulation of TORC1 activity upon amino acid limitation

*PIKK phosphoinositide 3-kinase related kinase.

regulate the TORC1 complex and its ability to bind the Fpr1-rapamycin complex [111], thus establishing another link between OS and aging. In *S. cerevisiae,* 15 genes are functionally related to TORC1 function and Tor, Sch9, and PKA signaling are interconnected (Table 1). In *S. cerevisiae,* the genes *TPK1* (alias *PKA1*, *SRA3*; ORF, YJL164C), *TPK2* (alias *PKA2*, *YKR1*, *PKA3*; ORF, YPL203W), and *TPK3* (YKL166C) encode for three forms of the cAMP-dependent protein kinase catalytic subunit of the cyclic AMP-dependent protein kinase (PKA) and *BCY1* (YIL033C) for the regulatory subunit. In *K. lactis,* homologous genes of main participants in these signaling pathways are present (Table 1) and homology (recorded using BLASTp in Génolevures at http://www.genolevures.org/) is summarized in Figure 2 The most remarkable observation is that in *K. lactis,* there is only one ORF (KLLA0B13948g), which encodes for a protein with 71% identity to *S. cerevisiae* Tor2 and 68% identity to *S. cerevisiae* Tor1. This opens a question about the existence and composition of two TORC complexes in *K. lactis* as previously reported in *S. cerevisiae* [110] and outlines this issue as a differential point to study in relation to divergences in life span signaling. Besides, the *K. lactis* proteins in this group with less than 40% identity to their *S. cerevisiae* counterparts are also good targets for further studies.

Apoptosis is one type of programmed cell death (PCD) with great importance for the development and homeostasis of multicellular organisms. Basal apoptosis machinery exists also in yeast, unicellular fungus, and in some filamentous

fungi [112]. Regarding the respiratory yeast *K. lactis*, once more the number of studies performed hitherto is very scarce [113]. A mutant in the essential gene *KlLSM4*, an ortholog to *LSM4* of *S. cerevisiae,* which encodes an essential protein involved in both pre-mRNA splicing and mRNA decapping, shows phenotypic markers of apoptosis such as chromatin condensation, DNA fragmentation, accumulation of ROS, and increased sensitivity to different drugs. Mechanisms of Bax-induced [114, 115] and lactose-induced [116] cell death have also been described in *K. lactis*. We have recently investigated PCD in *K. lactis*, using the drugs staurosporine (STS) and phytosphingosine (PHS), which induce PCD in other organisms, and found that glutathione and GLR played an important role. While *K. lactis* seemed to be insensitive to STS, PHS induced PCD. The insensitivity of *K. lactis* to STS might be dependent upon the higher levels of GSH found in cells treated with STS. In human cells, PCD induced by STS causes GSH efflux, but GSH exporter proteins are absent in *K. lactis*. In addition, GLR appears to be involved in PHS-triggered PCD because cells lacking this enzyme are more resistant to the drug than the wild-type strain. Moreover, the addition of GSSG or GSH to the medium partially restores growth of the wild-type *K. lactis* strain on PHS [117].

The strictly regulated removal of oxidized structures is a universal stress response of eukaryotic cells that targets damaged or toxic components for vacuolar or lysosome degradation. Autophagy stands at the crossroad between cell survival and death. It promotes survival by degrading proteins and organelles damaged during oxidative stress, but it is also activated as a part of death programs, when the damage cannot be overcome. Evidence is accumulating that the cellular sites of ROS production and signaling (including mitochondria) may be primary targets of autophagy [118]. The surplus ROS damage the mitochondria themselves and the damaged mitochondria produce more ROS in a vicious circle, ultimately leading to mitochondrial DNA deletion, a form of the so-called petite-mutant phenotype [119]. Selective mitochondria autophagy is called mitophagy and contributes to the maintenance of mitochondrial quality by eliminating damaged or excess mitochondria [120]. Although little is known about the mechanism, glutathione influences mitophagy [121, 122]. The interplay between mitochondria and autophagy seems to be evolutionarily conserved from yeast to higher eukaryotes. Defects in one of these elements could simultaneously impair the other, resulting in risk increments for various human diseases [123]. Autophagy is associated with tumor genesis, neurodegenerative diseases, cardiomyopathy, Crohn's disease, fatty liver, type 2 diabetes, defense against intracellular pathogens, antigen presentation, and longevity [121, 122, 124].

Recent studies in yeast identified several mitophagy-related proteins, which have been characterized with regard to their function and regulation, allowing to compare the similarities and differences of this degradation process between yeast and mammalian cells [120]. Up to our knowledge, no studies at all about mitophagy or even autophagy have been published at present in *K. lactis*. We have performed a custom Blastp in Génolevures for the main actors of mitophagy [125] and its signaling pathways [126] in *S. cerevisiae versus K. lactis* and we found sequences with different degrees of similarity as shown in Table 2 and Figure 2. Further research is required to analyze if the sequence similarity corresponds to similarity of function or not. This is a new field of research in the respiratory yeast *K. lactis*. As occurs with other pathways, it is likely that functional differences exist according to fermentative or respiratory predominant metabolism in yeasts. *K. lactis* proteins in this group (Figure 2) are good targets for comparative studies in mitophagy.

6. Conclusions and Perspectives

K. lactis is proposed as a respiratory eukaryote model, complementary to the fermentative *S. cerevisiae*, for the study of the pathways of hypoxia-induced oxidative stress. The experimental studies carried so far reveal that there are many differences in all the steps analysed from a comparative perspective, even when high homology exists between the acting proteins from the two yeasts. Some of these differences are briefly summarized in Table 3, although they are probably much wider than here exposed and they will increase with future studies. Besides, *in silico* analysis reveals that transcriptional factors and several actors from the cell-defense response (life span and mitophagy) are among the poorly homologous proteins, and therefore those become good candidates for functional characterization.

Many yeast genes related to the hypoxic, oxidative, and cell-defense responses are related to human diseases [127]. Although most of the studies performed hitherto about *K. lactis* physiology are focused on the respiro-fermentative metabolism, and much less is known about other pathways, there are representative examples of differences between *K. lactis* and *S. cerevisiae* that might be of interest for their applications in therapy of human health disorders and in the potential use of *K. lactis* as a model for this research. Among the potential genes or proteins of interest, *SOD1* is homolog of the human gene involved in amyotrophic lateral sclerosis [128]. NDI is involved in gene therapy of complex I defects [88, 129] and is important in neurological diseases [84–86]. Several genes of ergosterol biosynthesis are targets to look for pharmacological drugs (anticholesterol, antifungal, anticancer, etc.) [125]. Diamine oxidases and catalases have been used as therapeutic approaches for the treatment of inflammatory bowel diseases, intestinal cancers, or pseudoallergic reactions [130]. Hereditary coproporphyria (HCP), an autosomal dominant acute hepatic porphyria, results from mutations in the gene that encodes coproporphyrinogen III oxidase [131]. Sirtuins have been associated to diabetes type 2 [132] and Huntington disease [104] as well as cardiopathies or cancer [109].

Neither *S. cerevisiae* nor other currently used models, even multicellular, manifest the complex set of alterations associated to each health disorder in humans. This makes necessary the combination of the information obtained from several models, as representative as possible of the diversity of human cell types (*S. cerevisiae*, *K. lactis,* and others), in order to advance in puzzling out the molecular basis of the diseases and in developing new preventive and therapeutic tools.

Table 2: Putative main actors of mitophagy and its signaling pathways in *K. lactis* and *S. cerevisiae*. Degree of identity (%) between homologs is indicated in Figure 2.

Protein	ORF *K. lactis*	ORF *S. cerevisiae*	Function
Atg1	KLLA0C17160g	YGL180W	Autophagy-dedicated protein serine/threonine kinase
Atg11	KLLA0B12133g	YPR049C	Cytoplasm-to-vacuole targeting (Cvt) pathway and peroxisomal degradation (pexophagy)
Atg32	KLLA0A00660g	YIL146C	Mitochondrial receptor specific to mitophagy
Atg33	KLLA0A02695g	YLR356W	Detects or presents aged mitochondria for degradation at the stationary phase
Atg8	KLLA0E20593g	YBL078C	Component of autophagosomes and Cvt vesicles
Aup1	KLLA0F06985g	YCR079W	Mitochondrial protein phosphatase
Bck1	KLLA0F14190g	YJL095W	MAP kinase kinase kinase acting in the protein kinase C signaling pathway
Dnm1	KLLA0F12892g	YLL001W	Dynamin-related GTPase
Fmc1	KLLA0F04081g	YIL098C	Assembly at high temperature of mitochondrial ATP synthase
Hog1	KLLA0F20053g	YLR113W	MAP kinase involved in osmoregulation
Mdm38	KLLA0B11748g	YOL027C	Mitochondrial distribution and morphology
Mkk1	KLLA0D07304g	YOR231W	MAP kinase kinase acting in the protein kinase C signaling pathway
Mkk2	KLLA0D07304g	YPL140C	MAP kinase kinase acting in the protein kinase C signaling pathway
Pbs2	KLLA0E15313g	YJL128C	MAP kinase kinase in the osmosensing signal-transduction pathway
Pkc1	KLLA0E06447g	YBL105C	Protein kinase C
Rtg3	KLLA0E06513g	YBL103C	Transcription factor to activate the retrograde (RTG) and TOR pathways
Slt2	KLLA0B11902g	YHR030C	MPK1 MAP kinase
Uth1	KLLA0E14939g	YKR042W	Regulator outer membrane protein
Whi2	KLLA0F15972g	YOR043W	Full activation of the general stress response
Wsc1	KLLA0D14377g	YOR008C	Sensor transducer of the stress-activated PKC1-MPK1 kinase pathway
Yme1	KLLA0E06711g	YPR024W	Protease catalytic subunit for degradation of unfolded or misfolded mitochondrial gene products
Ynt1	KLLA0C06534g	YDR394W	Subunit of the 26S proteasome

Table 3: Main differences reported hitherto between *K. lactis* and *S. cerevisiae*, two alternative unicellular eukaryote models for hypoxic and oxidative stress responses.

	K. lactis	*S. cerevisiae*
Crabtree effect	Negative	Positive
Glucose catabolism in aerobic conditions	Mainly respiratory	Mainly fermentative
Ratio PPP/glycolysis for glucose catabolism	High	Low
Reoxidation of NADPH from PPP	Mainly by mitochondrial alternative external dehydrogenases	Mainly by cytosolic NADPH oxidoreductases
Catabolic repression of respiration	Low	High
Respiratory capacity	Unlimited	Limited
Petite phenotype	Positive in specific mutant genetic backgrounds	Positive
Caloric restriction increases longevity	No	Yes
Aerobic/hypoxic gene pairs	Absent	Present
Upregulated by hypoxia	Genes related to ergosterol synthesis, cell wall composition, and glycolytic genes. OS genes: *CUP1* and *CUP2*, *HSP12, FMP46* and *GRE1*, and *SOD1*.	Genes from the heme biosynthetic pathway, pyruvate decarboxylase, and lipid biosynthesis. OS genes: *KlOYE2, KlGSH1*. This response is highly dependent on the relative flux of glucose through glycolysis or PPP
Transcriptional regulators Hap1 and Rox1	Not related to heme-mediated oxygen response	Related to heme-mediated oxygen response

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