



Pharmacological models and approaches for pathophysiological conditions associated with hypoxia and oxidative stress



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ABSTRACT

Hypoxia is the failure of oxygenation at the tissue level, where the reduced oxygen delivered is not enough to satisfy tissue demands. Metabolic depression is the physiological adaptation associated with reduced oxygen consumption, which evidently does not cause any harm to organs that are exposed to acute and short hypoxic insults. Oxidative stress (OS) refers to the imbalance between the generation of reactive oxygen species (ROS) and the ability of endogenous antioxidant systems to scavenge ROS, where ROS overwhelms the antioxidant capacity. Oxidative stress plays a crucial role in the pathogenesis of diseases related to hypoxia during intrauterine development and postnatal life. Thus, excessive ROS are implicated in the irreversible damage to cell membranes, DNA, and other cellular structures by oxidizing lipids, proteins, and nucleic acids. Here, we describe several pathophysiological conditions and *in vivo* and *ex vivo* models developed for the study of hypoxic and oxidative stress injury. We reviewed existing literature on the responses to hypoxia and oxidative stress of the cardiovascular, renal, reproductive, and central nervous systems, and discussed paradigms of chronic and intermittent hypobaric hypoxia. This systematic review is a critical analysis of the advantages in the application of some experimental strategies and their contributions leading to novel pharmacological therapies.

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Abbreviations: BH2, dehydrobiopterin; BH4, tetrahydrobiopterin; CAO, cerebral artery occlusion; CAT, catalase; CKD, chronic kidney disease; COX, cyclooxygenase; CuZnSOD, superoxide dismutase; eNOS, endothelial nitric oxide synthase; ET-1, endothelin; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione oxidized; HIF, hypoxia-inducible factor; IH, intermittent hypoxia; IP, prostacyclin receptor; IR, ischemia reperfusion; NADPH, nicotinamide adenine dinucleotide phosphate; ONOO-, peroxynitrite; OS, oxidative stress; PGI₂, prostacyclin; PO₂, oxygen partial pressure; ROS, reactive oxygen species; XO, xanthine oxidase; VEGF, vascular endothelial growth factor; TBARS, thiobarbituric acid-reactive substances; PA, perinatal asphyxia.

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1. Introduction

Disorders characterized by hypoxia, such as myocardial infarction, stroke, peripheral vascular disease, and renal ischemia, are among the most frequent causes of morbidity and mortality (Foltynie & Kahan, 2013). Moreover, during development, chronic hypoxia may cause intrauterine growth restriction (IUGR) and markedly affect the functions of the newly developed organs (Fowden et al., 2006). Hypoxia is defined as the threshold where the oxygen concentration is a limiting factor for normal cellular processes since oxygen is an essential component for metabolism, including ATP synthesis. Furthermore, the integration of local responses defines hypoxia as a paradigm of reactions affecting the whole organism (Kwasiborski et al., 2012). Subsequently, an oxygen gradient arises between affected and non-affected tissues, stimulating the migration and proliferation of endothelial cells and fibroblasts, which intend to reconstitute normal oxygen supply by increasing perfusion (Nauta et al., 2014). If this process fails, a prolonged inadequate vascular supply of oxygen leads to chronic hypoxia and can cause chronic diseases. Further, intrauterine chronic hypoxia may increase the risk of developing cardiovascular disease later in life, with cardiovascular impairment and endothelial dysfunction (Giussani & Davidge, 2013).

Several difficulties exist in translating basic science findings into clinical practice. For instance, patients usually have marked differences in hypoxia or ischemia duration, concomitant disease, age diversity, co-morbidities, and the medications used. Therefore, accurately representing the clinical situation when establishing an animal model, is a challenge. Moreover, animal models need to account for the differences in response and species-specific reactions in relation to hypoxia (Garcia-Dorado et al., 2009). Despite these limitations, results from animal studies have allowed us to gain considerable insight into the mechanisms of specific phenomena, aiding the design of clinical trials using new pharmacological approaches. The major advantages of hypoxic animal models include highly reliable mechanistic experimental data and the conservation of responses among mammalians.

The findings from animal-based research can establish a cause-effect relationship between a hypoxic protocol and endpoints, such as, reduction in cell death, function improvement, and tissue structure maintenance. This review describes some models for mechanistic studies in pathophysiological states, where the main means of damage are the induction of hypoxia and oxidative stress (OS).

2. Hypoxia and oxidative stress

2.1. General concepts

There is a balance between the production of ROS and the antioxidant system in healthy individuals. When this balance is slightly tipped in favor of ROS, there is continuous low-level oxidative damage in the biological system. This redox imbalance also plays a major pathophysiological role in several clinical conditions associated with hypoxia, such as cardiovascular and neurological dysfunction (Rodrigo et al., 2013).

If the initial increase of ROS is relatively small and occurs in a short period of time, the antioxidant response may be adequate to manage the excess ROS and restore the original redox balance. This physiological response involves a slight increase and/or temporary shift in the intracellular thiol/disulfide redox status toward oxidative conditions (Droge, 2002). However, when the elevated ROS levels are persistent (chronic OS), pathophysiological conditions may arise. Noteworthy, the redox reaction rates and production of ROS is segmented in biological systems by subcellular compartmentation. Thus, as a biological defense, organelles that exhibit high redox reaction rates provide membrane-limited compartments (e.g., mitochondria has electrochemical mechanisms aimed at ATP production, peroxisomes are enriched in H₂O₂ linked metabolism), and both reductants and oxidants have different distributions among compartments. The thioredoxin redox system and reduced glutathione (GSH)/ glutathione oxidized (GSSG) system

are “redox buffers” that protect proteins from oxidation and to maintain the redox balance within the cell. These systems are extremely redox dynamic exhibiting different redox potentials depending on the compartmentation (e.g., mitochondria, cytosol, nucleus, endoplasmic reticulum, Golgi, lysosome, peroxisome, or extracellular space), as well as on the physiological/pathological state of the cell. Therefore, it is critical to define the key point of divergence between an organism that can reset the redox status to its original balance and an organism that cannot restore the original healthy condition.

In essence, oxidative stress is established when ROS generation overwhelms an organism's antioxidant capacity. Furthermore, OS has been defined as a disturbance in the pro-oxidant/antioxidant balance toward the former, leading to potential damage (Sies, 1991).

Therefore, oxidative stress can result from the following scenarios:

- i. *Diminished levels of antioxidants*, for instance, mutations affecting the activities of antioxidant defense enzymes, such as superoxide dismutase (CuZnSOD), catalase (CAT), or glutathione peroxidase (GSH-Px), or toxins that deplete antioxidant defenses. Many xenobiotics are metabolized by conjugation with reduced glutathione (GSH); high doses of these substances can deplete GSH and cause oxidative stress, even if the xenobiotic is not itself a generator of reactive species. Deficiencies in dietary minerals (e.g., Zn²⁺, Mg²⁺, Fe²⁺, Cu²⁺, and Se), which act as cofactors for several antioxidant enzymes, can also cause oxidative stress.
- ii. *Increased production of reactive oxygen species*. ROS are molecules, which are produced as a result of oxygen metabolism caused by mitochondrial function, nicotinamide adenine dinucleotide phosphate (NADPH), cyclooxygenase (COX), and xanthine oxidase (XO), among others. Since the rate of mitochondrial electron transfer is orders of magnitude greater than thiol-related oxidative reactions, this organelle produces high amounts of ROS. The occurrence of particularly high-reduced state of thioredoxin system in mitochondria (−360 mV, compared with −290 mV in nucleus and −270 mV in cytosol) preserves the “physiological insulation” of the cell from high-flux electron transfer systems. Cellular environmental alterations such as hypoxia and hyperoxia alter these enzymatic functions and lead to excessive ROS formation (Clanton, 1985). In addition, exposure of cells or organisms to decreased or elevated levels of radical superoxide (O₂^{•−}), exposure to other toxins that are oxidant species (e.g., NO₂) or are metabolized to generate reactive species (e.g., paraquat), or the excessive activation of “natural” systems that produce such species (e.g., the inappropriate activation of phagocytic cells in chronic inflammatory diseases) (Clanton, 1985; Halliwell & Whiteman, 2004). In addition, the supraphysiological activation of pro-oxidant enzymes such as NADPH oxidase and XO (through increasing oxidative conversion of xanthine dehydrogenase) or an increase in the bioavailability of transition metals in their redox active state (e.g., Cu⁺ and Fe²⁺) can lead to a general oxidant environment.

2.2. Alterations induced by hypoxia

The deprivation of oxygen and nutrients results in a series of abrupt biochemical and metabolic changes in various tissues (Zepeda et al., 2013). The absence of oxygen halts oxidative phosphorylation, leading to mitochondrial membrane depolarization, ATP depletion, and inhibition of cellular transportation. During the absence of oxygen, cellular metabolism switches to anaerobic glycolysis, resulting in the accumulation of lactate, which reduces intracellular pH. Consequently, the intracellular accumulation of protons activates the Na⁺-H⁺ ion exchanger, which pumps protons out from the cell in exchange for Na⁺ entry. Diminishing ATP during hypoxia impairs the function of the Na⁺/K⁺-ATPase, thereby exacerbating the intracellular Na⁺ overload. In response, the reverse activation of the Na⁺-Ca²⁺ ion exchanger results in overloading intracellular Ca²⁺ as the cell tries to expel Na⁺

(Hausenloy & Yellon, 2013). This is an important mechanism that can lead to osmotic stress in the cell, thus triggering cell death processes (Murrow & Debnath, 2013). Paradoxically, severe hypoxia may induce an increase in ROS production, particularly the generation of O_2^- by oxygen-dependent enzymes such as cytochrome c oxidase, NADPH oxidase, and uncoupled endothelial nitric oxide synthase (eNOS) (Clanton, 1985; Sylvester et al., 2012) (Fig. 1).

3. Pharmacological studies in hypoxic models

3.1. Perinatal hypoxia and oxidative stress

Prenatal hypoxia and OS have been associated with intrauterine growth restriction (Cosmi et al., 2011; Herrera et al., 2014a). The latter can account for ~5% of intrauterine restriction in lowland populations and up to 18% in high-altitude populations (Giussani et al., 2001; Keyes et al., 2003; Soria et al., 2013). Chronic hypoxia and/or OS during pregnancy increases not only perinatal morbi-mortality but also the long-term consequences, known as “fetal programming of adult diseases” or “developmental origins of health and disease—DOHaD” (Cosmi et al., 2011; Giussani et al., 2012; Herrera et al., 2014b). In fact, several studies have shown that hypoxia, oxidative stress, and placental insufficiency during gestation increases the risk of developing coronary artery disease, heart failure, hypertension, and type II diabetes, among other chronic diseases (Thornburg et al., 2010; Giussani et al., 2012; Hanson & Gluckman, 2014; Herrera et al., 2014b). The mechanisms underlying these effects are complex and most probably a mix of

physiological, pathophysiological, adaptive, and non-adaptive processes, with many of them controlled by epigenetic mechanisms (Casanello et al., 2014; Hanson & Gluckman, 2014). From this point of view, the earlier the intervention, the higher the plasticity and the more beneficial the effects (Hanson & Gluckman, 2014).

3.1.1. Fetal models

In the early 1900s, Sir Joseph Barcroft (1872–1947), a pioneer of modern fetal research, began studying the physiology of the developing fetus and generated considerable interest in fetal oxygen metabolism. Thereafter, an increasing number of researchers have investigated the mechanisms underlying the fetal response to acute and chronic hypoxia. In early studies, the main focus was on the cardiovascular, metabolic, and neurological responses to acute hypoxia. The development of the chronic catheterized fetal sheep model in the early 1960s led to considerable number of discoveries on how the fetus responds and adapts to hypoxia (Assali et al., 1962; Dawes, 1984). Currently, it is known that cardiovascular fetal responses to hypoxia are coordinated by the autonomic nervous system, unleashing a rapid peripheral vasoconstriction triggered by a carotid chemoreflex (Giussani et al., 1993) and mediated by increased sympathetic activity (Giussani et al., 1993; Bennet & Gunn, 2009). This induces a marked bradycardia and peripheral vasoconstriction leading to a decrease in oxygen consumption (Giussani et al., 2014). Thus, under these conditions, the blood flow is redistributed privileging cerebral circulation (Cohn et al., 1974; Ashwal et al., 1984). If hypoxia is sustained, fetal hormones liberated into the blood stream maintain the peripheral constriction (Perez et al., 1989; Fletcher et al., 2006).

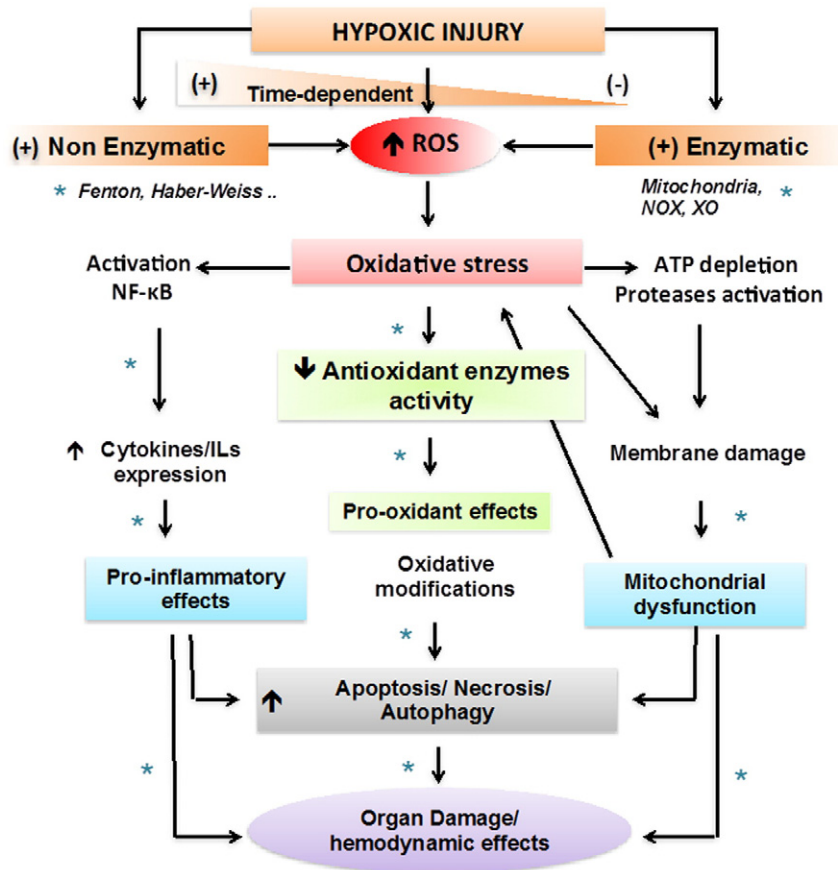


Fig. 1. Pathophysiology of hypoxic injury. This represents the cellular and molecular pathways of damage induced following time course of hypoxia. The activation of enzymatic and non-enzymatic sources of ROS is associated with the modulation of pro-inflammatory and pro-oxidant pathways, for example, the activation of NF-kappaB and the occurrence of mitochondrial dysfunction. ROS, reactive oxygen species; NOX, nicotinamide adenine dinucleotide phosphate-oxidase; XO, xanthine oxidase; ILs, interleukins (*) Target of antioxidants therapies, which are specified in Table 1.

Furthermore, there is now evidence of a local oxidant tone acting directly at the level of the fetal vasculature (Thakor et al., 2010a,b; Herrera et al., 2012; Kane et al., 2012; Giussani et al., 2014; Thakor et al., 2015). These responses have been characterized not only in lowland sheep but also in high-altitude-acclimatized animals studied either at lowlands (Longo et al., 1993; Giussani et al., 1999; Gilbert et al., 2003; Pena et al., 2007) or at high altitudes (Herrera et al., 2015a,b). Moreover, there are several studies in a high-altitude-adapted species, such as the llama, that show unique responses to hypoxia. These responses were presumably selected over several generations exposed to high-altitude environments (Giussani et al., 1999; Herrera et al., 2000; Llanos et al., 2002, 2003). One of the impressive features of fetal llama adaptation is marked peripheral vascular resistance and cerebral hypometabolism during an acute hypoxic episode (Llanos et al., 2003; Ebensperger et al., 2005), with no evidence of neuronal damage. All of the above fetal responses to hypoxia, either in adapted, acclimatized, or those not exposed to high altitude, developed a strategy for conserving oxygen supply for vital organs (Giussani et al., 2014) and activating cellular compensatory mechanisms (Herrera et al., 2015a,b) during O₂ shortage. Other models involving small rodents have been extremely useful in developing knowledge on the molecular mechanisms involved in the responses, adaptations, and the long-term consequences of fetal hypoxia (for reviews, see (Vuguin, 2007; Giussani & Davidge, 2013; Jang et al., 2015).

3.1.2. Pharmacological approaches

Over recent decades, researchers have searched for procedures or drug interventions to decrease the devastating impacts of hypoxia during development. One of the important mechanisms involved in hypoxia-induced systemic and cellular damage is oxidative stress and excessive ROS generation (Giussani & Davidge, 2013; Herrera et al., 2014a). Controlling ROS at the cellular level is essential for normal fetal cardiovascular function (Thakor et al., 2010a,b; Herrera et al., 2014a). For instance, superoxide anion combines with NO to promote an oxidant tone contributing to endothelial dysfunction in fetal circulation. This oxidant tone contributes to the maintenance of basal total peripheral vascular resistance and arterial blood pressure in the fetus. It also contributes to the maintenance of umbilical blood flow and the fetal brain sparing response to acute hypoxia (Gardner & Giussani, 2003; Herrera et al., 2012; Kane et al., 2012). Therefore, most novel treatments are based on their antioxidant and/or vasodilator capacities for decreasing ROS-induced alterations and improving feto-placental perfusion (Herrera et al., 2012; Kane et al., 2012; Giussani et al., 2014; Kane et al., 2014; Thakor et al., 2015). However, these proposed treatments are still in the animal research field, as it is controversial whether potentiation or decreasing the fetal response to hypoxia is the best way to approach a hypoxic fetus. Another evidently limiting condition is the fact that the fetus is isolated in the womb. Therefore, proposed strategies are to administer the treatments to the mother using antioxidants such as vitamins C and E, allopurinol, and melatonin, among others (Poston et al., 2006; Buonocore & Groenendaal, 2007; Poston et al., 2011; Miller et al., 2012; Kaandorp et al., 2014; Miller et al., 2014; Kaandorp et al., 2015). In fetal experimental models, the uterine, placental, and umbilical vasculature during pregnancy are highly sensitive territories to changes in pO₂ and ROS levels (Richter et al., 2009; Richter et al., 2012; Herrera et al., 2014a). Although, these experimental evidences have suggested the rationale for antioxidant therapy during pregnancy, there are still no effective treatments. Several randomized controlled trials have been performed to determine whether antioxidants supplementation in complicated pregnancies are beneficial, showing no evidence that these supplements may prevent hypoxia- or ROS-induced damage (Poston et al., 2006; Basaran et al., 2010; Roberts et al., 2010; Xu et al., 2010; Conde-Agudelo et al., 2011; Kalpdev et al., 2011; Poston et al., 2011; Rumbold et al., 2011; Polyzos et al., 2012). In marked contrast, under highly controlled conditions, the use of different antioxidants in animal models was highly effective in

preventing pregnancy complications, as well as short- and long-term impairments. The debate is still open and further studies need to indicate when and how the antioxidant intervention is pertinent.

3.1.3. Neonatal models

Prolonged and acute hypoxia are the most frequent conditions in neonatal complications, with devastating immediate and long-lasting effects (Paolo, 2012). As the CNS is a highly sensitive system to pO₂ changes, the most frequent conditions associated with perinatal hypoxia are neonatal hypoxic/ischemic encephalopathy leading to cerebral palsy. Furthermore, perinatal hypoxia disrupts normal brain development, potentially leading to neurodevelopmental deficits such as motor and sensory abnormality, learning disability, mental retardation, and seizure attacks (Herrera-Marschitz et al., 2014). Another exquisitely sensitive territory to low oxygen is the pulmonary circulation, where a decrease in pO₂ leads to pulmonary hypertension ending in endothelial dysfunction, vascular remodeling, and heart failure (Lakshminrusimha, 2012; Herrera et al., 2015a,b). Furthermore, chronic perinatal adverse conditions and/or hypoxia are closely associated with increased risks of development of hypertension, coronary heart disease, type 2 diabetes, obesity, hyperlipidemia (Barker et al., 1993; Hanson & Gluckman, 2014).

To distinguish the various mechanisms involved in the neurological disorders and cardiovascular impairments due to perinatal hypoxia, a variety of animal experimental models have been developed, ranging from small rodents to large animals.

3.1.4. Perinatal asphyxia: brain effects

Perinatal asphyxia (PA) is defined as the temporal interruption of oxygen availability (hypoxia) and gas exchange (O₂/CO₂) to the fetus/neonate resulting in hypoxemia and hypercapnia in vital organs, including the brain (Kapadia et al., 2013; Herrera-Marschitz et al., 2014). The initial cerebral hypoxic and/or ischemic insult leads to a switch from aerobic metabolism to glycolysis, resulting in severe energy depletion. The reoxygenation process generates a secondary injury due to a wave of further energy failure and the formation of free radicals, proteases, and caspases, which lead to neuronal damage (Seidl et al., 2000; Herrera-Marschitz et al., 2011, 2014).

A model to investigate the outcome of PA in rats was proposed by Bjelke et al. (1991). In this model, global asphyxia is induced at the time of birth mimicking relevant aspects of human labor and delivery. It is non-invasive and allows studying, with high reproducibility the short- and long-term effects of PA. In this model, a pregnant rat, in its last day of gestation, is anesthetized, euthanized by neck dislocation, and hysterectomized. The uterine horns containing fetuses are removed and immersed in a water bath at 37 °C for increasing times (0–22 min). Subsequently, the uterine horns are incised, and the pups are removed and stimulated to breathe. After a period of recovery and evaluation of respiratory, cardiovascular, and neurological parameters, the pups are given to surrogate mothers until further analysis (Bjelke et al., 1991; Herrera-Marschitz et al., 2011). The Apgar evaluation is a critical parameter in order to identify the severity of PA (Bacigaluppi et al., 2010).

PA occurs at a high rate when delivery is prolonged, despite improvements in perinatal care (Herrera-Marschitz et al., 2011; Douglas-Escobar & Weiss, 2015). The incidence has been reported as 1–6/1000 live births in developed countries, reaching higher rates in underdeveloped countries (Kurinczuk et al., 2010; Douglas-Escobar & Weiss, 2015).

The time course and the severity of the neurological sequelae observed following PA depend on the extent of the insult, the time lapse before normal breathing is restored and the CNS maturity of the fetus (Vannucci & Hagberg, 2004). Severe asphyxia has been linked to cerebral palsy, mental retardation, and epilepsy, while mild–severe asphyxia has been associated with attention deficit/hyperactivity disorder in children and adolescents (Maneru et al., 2001, 2003) and an increased risk for low intelligence quotient score (Odd et al., 2009). Obstetric

complications are also the key risk factors of psychiatric diseases, including, schizophrenia (Lou, 1996; Cannon et al., 2008; Odd et al., 2009; Sommer et al., 2010; Morales et al., 2011), bipolar disorders (Haukvik et al., 2014), and vulnerability to drug abuse, in particular to cocaine addiction (Galeano et al., 2013).

There is clinical and experimental evidence indicating that the neuro-circuitries of the basal ganglia and hippocampus are particularly vulnerable to neonatal hypoxia/ischemia (Morales et al., 2003, 2008, 2011). The hippocampus is associated with specific cognitive functions such as memory and attention, and together with the striatum, it plays a role in the pathogenesis of attention deficit/hyperactivity disorder, autism, and schizophrenia (Lou, 1996). The striatum is also associated with cerebral palsy, disorders of movement, and posture (Ferrari et al., 2011).

3.1.5. PA and energetic metabolism

A delay in starting pulmonary ventilation at birth results in a switch to glycolysis, which is a poor metabolic alternative for neurons. Neonatal brain tissue has low stores of glucose, aggravating the energetic crisis (Wyss et al., 2011; Brekke et al., 2015). Pyruvate accumulation leads to lactate accumulation. Lactate provides low energy levels and also leads to acidosis (Chen et al., 1997). All these changes imply partial recovery and sustained over expression of alternative metabolic pathways, prolonging the energy crisis. In response to the energy deficit, blood flow is redistributed to vital organs such as the heart, brain, and adrenal glands. This redistribution occurs at the expense of the kidneys, gastrointestinal tract, muscles, skeleton, and skin (Herrera-Marschitz et al., 2014). There is also a local redistribution of blood flow in the brain, favoring the brain stem at the expense of the neocortex, showing a re-compartmentalization to privilege survival (Herrera-Marschitz et al., 2011).

The depletion of ATP results in neuronal depolarization and the release of excitatory amino acids into the extracellular space, exceeding both the glial reuptake capacity and the reuptake into the synaptic nerve terminal by impairment of Na^+ , K^+ -ATPase-dependent pumps, reaching excitotoxic levels (Morales et al., 2011). This excitotoxic environment (Olney, 1969; Benveniste et al., 1984) further activates proteases, lipases, endonucleases, and nitric oxide synthases that finally lead to membrane lipid peroxidation and the accumulation of peroxynitrites and other free radicals species (Benveniste et al., 1984).

Reoxygenation is a requirement for survival, but it further increases ATP consumption, extracellular glutamate levels, Ca^{2+} conductance, phospholipases activation, and lipid peroxidation. This leads to an increase in the toxic effects of NO leading to the OS (Kapadia et al., 2013), increasing pro-inflammatory signaling (NF- κ B) (Neira-Peña et al., 2015), DNA sensing proteins (poly(ADP-ribose) polymerase, PARP-1) (Bustamante et al., 2003; Allende-Castro et al., 2012; Neira-Peña et al., 2015), and astrocyte reactivity, which in turn induce cell death (Herrera-Marschitz et al., 2014). Furthermore, asphyxia induces long-term effects in neuronal branching (Morales et al., 2003; Klawitter et al., 2007; Rojas-Mancilla et al., 2015), synaptogenesis (Rojas-Mancilla et al., 2015), synaptic plasticity (LTP), and learning (Dell'Anna et al., 1997; Morales et al., 2010; Neira-Peña et al., 2015; Rojas-Mancilla et al., 2015).

In comparison to the mature brain, the immature brain is particularly vulnerable to OS because of its high oxygen consumption, high metabolic rate (by three to seven times) (Brekke et al., 2015), low mitochondrial density, low concentrations of mitochondrial proteins and respiratory enzymes (Erecinska et al., 2004), low antioxidant defenses, high oxidative phosphorylation, high free iron producing hydroxyl radicals, fatty acid content (Dringen, 2000; McQuillen & Ferriero, 2004), and the low and immature blood–brain barrier. This makes the immature brain susceptible to infiltration of toxic elements produced either by anaerobic metabolism or inflammatory pathway activation, which occurs locally in the brain or in the periphery (Golubnitschaja et al., 2011; Hagberg et al., 2014).

3.1.6. Pharmacological targets for neonatal hypoxia–ischemia (HI) neuroprotection

3.1.6.1. Hypothermia. Hypothermia treatment has been established as a therapeutic tool for term babies with PA or HI in developed countries (Edwards et al., 2010; Perlman et al., 2010; Chevallier et al., 2013; Wu et al., 2014), showing an overall 25% reduction in death and/or neurological damage at 18 months (Edwards et al., 2010), with the consequent improvement of neurocognitive outcomes in surviving children at 6–7 years old (Azzopardi et al., 2014). The rationality for hypothermia is the graded reduction of cerebral metabolism—about 5% for every degree of temperature reduction (Erecinska et al., 2003). Whole-body hypothermia and selective head cooling have been used with equal effectiveness (Tagin et al., 2012). The time to initiate hypothermia correlated with the outcomes. Neonates undergoing earlier cooling therapy (within 180 minutes of birth) had better outcomes compared with those who underwent the therapy later (180–360 minutes after birth) (Thoresen et al., 2013). The protective mechanisms that underlie therapeutic hypothermia are multifactorial (Wassink et al., 2015). However, investigations to establish adjuvant neurotherapies are ongoing, to diminish the risk of death and brain injury despite treatment with hypothermia (Coleman et al., 2013; Azzopardi et al., 2014; Massaro et al., 2015).

3.1.6.2. PARP-1 Inhibition. PARP-1 is a sentinel proteins involved in the maintenance of chromatin integrity, recruiting the DNA repair machinery through poly-ADP ribosylation of histones, relaxing chromatin, and modulating different factors with high affinity for pADPr (Virag & Szabo, 2002). It has been shown that neonatal hypoxia induces PARP-1 hyperactivity worsening metabolic failure and depleting further NAD^+ availability leading to caspase-independent apoptosis, via the translocation of mitochondrial pro-apoptotic proteins (Virag & Szabo, 2002). PARP-1 overactivation also leads to an NF- κ B signaling cascade, resulting in I κ B α degradation and nuclear p65 translocation. This increases TNF- α and IL-1 β transcription, apoptotic-like cell death, and microglial migration, toward the site of neuronal injury (Neira-Peña et al., 2014, 2015). Thus, it is hypothesized that PARP-1 is involved in the long-term effects produced by PA (Herrera-Marschitz et al., 2011; Morales et al., 2011), making it relevant to monitor PARP-1 levels and PARP-1 activity at early stages, when babies are recovering from PA. PARP-1 inhibition is a proposed target for neuroprotection following PA (Herrera-Marschitz et al., 2011, 2014; Neira-Peña et al., 2015). Several PARP inhibitors are shown to decrease both brain damage and inflammation, improving the neurological outcome of perinatal brain injury (Jagtap & Szabo, 2005). Nicotinamide is an NAD^+ precursor in mammals, functioning as a coenzyme of cellular metabolism, with a role in redox reactions (Galli et al., 2010). Nicotinamide counteracts PARP-1 overactivation (Virag & Szabo, 2002), replacing NADH/NAD^+ (Zhang et al., 1995), and protects against oxidative stress (Sakakibara et al., 2000), inflammation (Ducrocq et al., 2000), and cell death, even if the treatment is delayed for 24 h, suggesting a clinically relevant therapeutic window (Herrera-Marschitz et al., 2014; Neira-Peña et al., 2015).

The relative low potency of nicotinamide has an advantage when used with developing animals because it can antagonize the effects elicited by PARP-1 overactivation without impairing DNA repair. Thus, nicotinamide prevents several short- and long-term outcomes elicited by PA, evaluated at neurochemical (Bustamante et al., 2007), cellular (Klawitter et al., 2007), and behavioral (Simola et al., 2008; Morales et al., 2010) levels. Systemic neonatal nicotinamide administration also prevents activation of pro-inflammatory signaling and cell death (Morales et al., 2010; Neira-Peña et al., 2015). Nicotinamide has already been tested in human clinical trials, but not for other clinical indications (Macleod et al., 2004).

3.1.6.3. Stem cells and trophic factors. Recent advances suggest that neurotrophic factors and/or stem cell transplantation may improve

repair of the HI-induced brain damage. Neurotrophic factors including bFGF (Russell et al., 2006), insulin growth factor-1 (IGF-1) (Gluckman et al., 1992; Zhong et al., 2009), nerve growth factor (NGF) (Holtzman et al., 1996), and brain-derived neurotrophic factor (BDNF) (Han & Holtzman, 2000) were found to reduce long-term HI-induced brain damage and improve behavioral recovery in immature rats. Several types of stem cells including neuronal stem cells (NSC), mesenchymal stem cells (MSC) (van Velthoven et al., 2011; Cameron et al., 2015; Park et al., 2015; Sun et al., 2015), and hematopoietic stem cells (HSC) were transplanted to both neonatal and adult animal models of HI, promoting functional as well as anatomical recovery. Regenerative effects of stem cell transplantation involve both replacements of damaged cells by exogenous cells, as well as the improvement of endogenous repair processes by releasing trophic factors (Wakabayashi et al., 2010). A single hemispheric injection with MSC, 7–10 days after HI, induced an upregulation of genes involved in cell survival, proliferation, and neurogenesis. Two injections of MSC induced the expression of genes involved in cell proliferation, differentiation, and network integration in the injured brain (van Velthoven et al., 2011). Recently, it was shown that a 7-day delay in subcutaneous and intraperitoneal MSC administration, at either a high or low doses, restores striatal medium-spiny projection neurons, improving motor function following neonatal HI injury (Cameron et al., 2015). Intraperitoneal transplantation of human umbilical cord blood mononuclear cells (HUCB), 3 h after the HI insult, resulted in a neuroprotective effect in the striatum and a decrease in the number of activated microglial cells in the cerebral cortex, suggesting that HUCB transplantation might rescue striatal neurons from cell death after neonatal HI injury, resulting in better functional recovery (Pimentel et al., 2015). Combined hypothermia/HUCB treatment improves the prognosis of severe HI (Park et al., 2015). Recently, the potential use of stem/progenitor cell therapies for neuroprotection or regeneration after neonatal HI was evaluated in several preclinical studies, and the most promising results are now being tested in clinical trials (Liao et al., 2013; Cotten et al., 2014; Gunn & Thoresen, 2015).

In summary, emerging pharmacological targets for early intervention and neuroprotection are focused on the inhibition of various potentially destructive molecular pathways including excitotoxicity, inflammation, oxidative stress, and cell death. Furthermore, therapies are targeted to restoring functionality of neuro-circuitries by stimulating neurotrophic endogenous properties of the neonatal brain using neurotransmitter agonist or antagonist, growth factors, and stem cell transplantation. The use of these novel interventions alone or in combination is very attractive and needs further research. It is expected that future studies will allow the identification of critical molecular, morphological, physiological, and pharmacological parameters, specifying variables that should be considered when planning neonatal care and development programs.

3.1.7. Perinatal hypoxia and oxidative stress: pulmonary effects

The most commonly used model system for testing pulmonary hypertension in neonates are mid- to large-sized animals, such as piglets and lambs (Naeije & Dewachter, 2007; Papamatheakis et al., 2013a,b). The main reasons for the use of these species are their comparable similar cardiopulmonary physiology and easily accessible to hemodynamic variables, *in vivo*.

An adequate pulmonary circulation is established within a few minutes after birth, where it must change from a high vascular resistance/low blood flow in the fetus (8–10% of combined cardiac output) to a low vascular resistance/high blood flow circulation (100% cardiac output) (Rudolph, 1979; Abman, 2007). This transition is dependent on several mechanisms at birth, of which the most relevant are (i) increased pO_2 , (ii) initiation of pulmonary ventilation that allows the alveolar distention, (iii) vascular shear stress, and (iv) release of potent vasodilator mechanisms, such as NO and prostacyclin (PGI_2) (Abman, 2007). The failure of establishing a normal decrease in pulmonary

vascular resistance (PVR), at birth results in pulmonary hypertension of the neonate (PHN). Therefore, pulmonary hypertension can be induced by chronic hypoxia, ductal ligation, and pulmonary hypoplasia (van Loenhout et al., 2009; Papamatheakis et al., 2013a,b). Pulmonary circulation has an intrinsic unique response to hypoxia, where low oxygenation induces arterial constriction, known as hypoxic pulmonary vasoconstriction (HPV). Although the specific mechanisms are still to be discovered, hypoxia induces intracellular Ca^{2+} increase, presumably by activation of K^+ and Ca^{2+} channels (Aaronson et al., 2006). This response is fast in its onset and easily reverted with oxygenation (Aaronson et al., 2006). However, persistent HPV response leads to structural and functional changes in the pulmonary vasculature, leading to pulmonary hypertension and right heart remodeling (Papamatheakis et al., 2013a,b). In fact, chronic hypoxia induces several growth factors such as HIF-1 and VEGF (Grover et al., 2003; Shimoda & Semenza, 2011). In addition, not only oxygen deprivation, but mechanical stretching and ROS increases HIF activity both conditions present in PHN (Pouyssegur & Mechta-Grigoriou, 2006; Gorchach & Kietzmann, 2007; Wedgwood et al., 2015). HIF-1 α stabilizes under hypoxia and translocates to the nucleus, initiating the transcription of hundreds of different target genes (Semenza, 2014). This activation of transcription promotes SMC proliferation, vasoconstrictor-enhanced synthesis, and arterial lumen narrowing (Semenza, 2014; Veith et al., *in press*) via vascular remodeling genes, such as COX-2, endothelin-1 (ET-1), heme oxygenase-1 (HO-1), VEGF, and iNOS (Schofield & Ratcliffe, 2004; Semenza, 2006).

Conversely, animal models showed that chronic hypoxia induces a marked endothelial dysfunction associated to the impaired function of vasodilator pathways. Some of them paradoxically enhanced eNOS expression but decreased nitric oxide function due to impairments in the NO-cGMP pathways (Herrera et al., 2007, 2008a,b; Llanos et al., 2011; Torres et al., 2015). In addition, the NO function also decreases due to its high reactivity with superoxide anion, generating peroxynitrite and decreasing NO bioavailability (Huie & Padmaja, 1993; Tabima et al., 2012; Torres et al., 2015). Simultaneously, there is an increased expression of phosphodiesterase V (PDE5), enzyme that opposes NO-dependent vasodilatation by degrading cGMP (Rybalkin et al., 2003; Herrera et al., 2008a,b). Furthermore, an enhanced NO function (as shown in high-altitude lambs) increases cGMP and its binding to the GAF domains, which directly activates PDE5 catalytic activity (Rybalkin et al., 2003). Moreover, increased cGMP (via NO) and cAMP (via PGI_2) in hypoxia may also upregulate PDE3 and activate PDE5 in pulmonary hypertensive arteries (Murray et al., 2011). The upregulation of PDE3 might be induced not only by the excess of cAMP but also by the nuclear factor kappa B (NF- κ B), a transcription factor that increases in oxidative stress conditions. Ultimately, these mechanisms tend to impair the main vasodilator pathways in the neonatal lung due to hypoxia and oxidative stress. Hence, the therapeutic targeting of more than one molecule or cellular pathway seems to be necessary to revert PHN.

3.1.7.1. Pharmacological targets for pulmonary hypertension. Hypoxia and oxidative stress appears as important mediators in pathological mechanisms in PHN, inducing several cellular and systemic responses. These responses end in endothelial damage and excessive proliferation of vascular smooth muscle (Sylvester et al., 2012; Tabima et al., 2012). Therefore, several research groups have not only proposed vasodilator drugs for PHN, but novel approaches predicted to restore appropriate levels of oxygen and reactive oxygen species. Currently, assisted ventilation and inhaled nitric oxide (iNO) are the only treatments approved by the U.S. Food and Drug Administration (FDA) for the treatment for pulmonary hypertension of the newborn and severe respiratory failure (Roberts et al., 1997; Clark et al., 2000). Although, iNO effectively increases oxygenation (Finer & Barrington, 2006), 30–50% of neonates fail to respond to this treatment (NINOS Group, 1997; Roberts et al., 1997; Field et al., 2005; El-Ferzli et al., 2012). Other treatments have been proposed with sildenafil, a PDE-5 inhibitor, with potentially good results in

terms of oxygenation and pulmonary pressure decrease (Baquero et al., 2006; Herrera et al., 2008a,b). However, a large-scale randomized trial comparing sildenafil with the currently used vasodilator is needed to assess efficacy and safety (Shah & Ohlsson, 2011). Attempts for other new treatments are still under experimentation. For instance, milrinone, a phosphodiesterase 3 inhibitor, which induces pulmonary vasodilatation by acting through a cAMP-mediated signaling pathway, improves oxygenation in patients with suboptimal response to iNO (McNamara et al., 2013). The same can be said regarding the use of prostaglandins (PGI₂) (Porta & Steinhorn, 2012), thromboxane inhibitors (Bendapudi et al., 2015), and bosentan, an antagonist of the ET-1 receptors (Nakwan et al., 2009); however, randomized controlled trials are needed to confirm experimental findings (Bendapudi et al., 2015). All these treatments are based on direct vascular effects aimed at either activating vasodilator pathways or decreasing vasoconstrictor ones (Steinhorn, 2013). However, pulmonary hypertension is a complex syndrome that also involves oxidative damage and remodeling processes. Therefore, some studies were performed with antioxidants such as melatonin, allopurinol, and vitamins C and E (Miller et al., 2012). For instance, the oral neonatal administration of melatonin is able to improve the pulmonary oxidant tone, decrease oxidative stress, and recover endothelial function (Torres et al., 2015). Furthermore, the blockade of store-operated calcium channels also showed important effects on pulmonary vascular resistance during hypoxia (Parrau et al., 2013). Attempts to decrease the activation of HIF-1 α to diminish remodeling processes and vasoconstrictor increments are also interesting approaches (Veith et al., in press). However, these have not been tested in neonates. Based on current knowledge, it is very likely that the use of therapies acting on a single pathway will not improve the current treatments for PHN. A critical analysis of the literature indicates that a potential treatment is a combination of low doses of pulmonary vascular vasodilators, antiproliferative, and antioxidant agents. However, drug interactions need to be assessed before clinical trials.

3.2. Hypoxia and oxidative stress in adults

3.2.1. Cardiovascular system

The cardiovascular system is constantly coping with organismal and cellular levels of hypoxia and is the principal system that optimizes oxygen delivery to the organism. Under physiological conditions, it can usually satisfy body demands. However, under pathological state, local perfusion may be affected by thromboembolism or vessel occlusions, for example. Furthermore, the cardiovascular responses frequently prioritize vital functions through perfusions, despite promoting local oxidative stress in perfused hypoxic organs.

3.2.1.1. Cardiac hypoxia. Cardiac hypoxia is the result of a disproportion between oxygen supply and demand. Due to high coronary arteriovenous differences, the myocardium is unable to bring about substantial improvements in oxygen supply by increasing extraction of oxygen from the blood. Thus, the only way of meeting the higher oxygen demand is by increasing blood supply. Theoretically, any of the known mechanisms leading to tissue hypoxia could be responsible for a reduction of oxygen supply in the cardiac tissue. However, the most common causes are undoubtedly (i) ischemic hypoxia (often described as “cardiac ischemia”) induced by the reduction or interruption of the coronary blood flow and (ii) systemic (hypoxic) hypoxia (“cardiac hypoxia”) characterized by a fall in pO₂ levels in the arterial blood, but with adequate perfusion (Clanton, 1985; Sylvester et al., 2012). The effects of ischemia are usually more severe than hypoxia and typically include lactic acidosis due to anaerobic glycolysis, impaired mitochondrial energy production and cell death (Essop, 2007).

Hypoxia is a major contributor to cardiac pathophysiology, including myocardial infarction, cyanotic congenital heart disease, and chronic cor pulmonale (Budev et al., 2003). Chronic lack of oxygen leads to an increase in pulmonary vasoconstriction, in order to redistribute

pulmonary blood flow from low to high PO₂ regions (Hislop & Reid, 1976). However, chronic pulmonary vasoconstriction may result in pulmonary hypertension, increasing the after load on the right ventricle, which may eventually lead to heart failure.

Although the pathophysiological effects of hypoxia reveal a complex interplay between signaling cascades, intracellular mediators, and sub-cellular organelles such as the mitochondrion (Solaini & Harris, 2005), the regulatory mechanisms underlying the cardioprotective effects of physiological hypoxia are less well understood. Here, greater insight may reveal novel therapeutic strategies for the treatment of heart disease.

3.2.1.2. Cardiac intermittent hypoxia. Intermittent hypoxia (IH) is broadly defined as repeated episodes of hypoxia interspersed with episodes of normoxia. The actual protocols that are experimentally vary greatly in cycle length, the number of hypoxic episodes per day, and the number of exposure days (Neubauer, 1985). Intermittent hypoxia is an effective stimulus for evoking respiratory, cardiovascular, and metabolic adaptations normally associated with continuous chronic hypoxia (Weil, 1986). These adaptations are thought by some to be beneficial in that they may provide protection against the disease, as well as improve exercise performance in athletes (Almendros et al., 2014). The long-term consequences of chronic intermittent hypoxia (CIH) may have detrimental effects, including hypertension, cerebral, and coronary vascular problems, developmental and neurocognitive deficits, and neurodegeneration due to the cumulative effects of persistent bouts of hypoxia (Harper et al., 2013).

Several studies have reported that IH-induced adaptations or intermittent hypoxic training can provide some measurable protection in some disease states or enable improvements in selected sport-related performances (Katayama et al., 2003; Almendros et al., 2014). The protective effects of IH can be explained by the activation and propagation of homeostatic or adaptive responses elicited by the IH stimulus, usually through a process that has been generally termed preconditioning. Thus, short exposures to mild IH paradigms can afford protection to specific cells, tissues, or organs against more severe hypoxia and ischemia (Zhang et al., 2012).

Animals subjected to various paradigms of acute IH become more resistant to the injury induced by subsequent exposures to severe hypoxic/ischemic insults. For instance, compared to controls, mice treated with brief episodes of low-frequency IH (8% O₂ \times 10 min/21% O₂ \times 10 min, 6 cycles) survived substantially longer than those exposed to lethal hypoxia. This phenomenon was accompanied by attenuated cellular and tissue injury to crucial organs, such as the lung and the brain. Furthermore, myocardia from mice treated with a similar IH paradigm (6% O₂ \times 6 min/21% O₂ \times 6 min, 5 cycles) or from rats treated with an IH paradigm with higher frequency but short duration (10% O₂ \times 40 s/21% O₂ \times 20 s, for 4 h) were protected against ischemia-induced infarction (Beguín et al., 1985; Cai et al., 2003). Such IH-induced cardioprotection seemed to involve the activation of pathways similar to those described in models of ischemic cardiac preconditioning (Cai et al., 2003; Park et al., 2007), in which sufficient expression and activity of hypoxia-inducible factor 1 α (HIF-1 α) is required (Cai et al., 2003). IH can protect the heart against ischemia-reperfusion (IR) injury by improving ischemia-induced contractile dysfunction (Chen et al., 2006), endothelial dysfunction (Manukhina et al., 2013), arrhythmias (Meerson et al., 1987; Manukhina et al., 2013), and cell death (Kolar et al., 2005). Also, IH treatment can promote higher resistance to arrhythmias during acute myocardial ischemia and prevent the development of atherosclerosis in rabbits (Kitaev et al., 1999). This protection has been ascribed to higher myocardial vascularity, coronary blood flow, cardiomyoglobin, and expression of antioxidant proteins induced by IH (Zhuang & Zhou, 1999). In addition, IH appears to provide a therapeutic effect on permanent coronary artery ligation-induced myocardial infarction by reducing the infarct size, myocardial fibrosis, and apoptosis (Xu et al., 2011). Due to the benefits reported by IH on myocardial infarction and that IH

treatment is simple, non-invasive, and has limited scarce adverse effects, IH paradigms have been proposed as treatment for patients suffering from heart diseases.

3.2.1.3. Models of cardiac hypoxia. A constant supply of oxygen is indispensable for cardiac viability and function. However, the role of oxygen and oxygen-associated processes in the heart is complex, and they can either be beneficial or contribute to cardiac dysfunction and death. As oxygen is a major determinant of cardiac gene expression, important in ROS formation and numerous other cellular processes, it is essential to understand its role in the heart in order to elucidate the pathogenesis of cardiac dysfunction.

The normal heart is an extremely versatile organ capable of adapting itself to a wide range of functional requirements (Baker et al., 1995). Increasing workload demands leads to local tissue hypoxia and depletion of energy stores with an accumulation of metabolites that, in turn, induce or depress the protein synthesis process (Azar et al., 2003). Ischemia and chronic hypoxia, secondary to coronary artery disease, are often associated with hypertrophy (Cervos et al., 1999). Hypoxia was shown to promote the formation of new capillaries in heart muscle, to stimulate growth in tissue cultures and to stimulate RNA synthesis in the perfused heart. If hypoxia is the stimulus for adaptive cardiac growth, it must operate over a narrow range of oxygen tension since severe hypoxia with concomitant reduction in energy stores was been shown to inhibit, rather than stimulate, protein synthesis in the isolated perfused heart and isolated atria (Cervos Navarro et al., 1999; Chen et al., 2014). Also, the response of the heart to increased workload depends on the age of the animal at the time of stress is imposed (Oparil et al., 1984; Kajstura et al., 1994).

In the case of normobaric hypoxia, obstructive sleep apnea (OSA) is a common chronic sleep disorder. OSA-related high-frequency intermittent normobaric hypoxia (IH) is characterized by cycles of hypoxemia with reoxygenation that is distinctly different from sustained low-frequency hypoxia, and contributes to cardiac hypoxic injury and cardiovascular dysfunction. For example, the relationship between OSA and high blood pressure may be linked through endothelial mechanisms. These include dysfunction of vasoconstrictor (e.g., endothelin) and vasodilator (e.g., nitric oxide) endothelial factors, abnormalities in the renin–angiotensin–aldosterone system, or circulating levels of atrial natriuretic peptide (Phillips et al., 1999). Indeed, nocturnal IH may be a contributing factor to increases in muscle sympathetic nervous system activity and cardiovascular dysfunction. However, the effect may be absent in cases of mild and moderate hypoxemia (Lam et al., 2014). Likewise, absent or minimal cardiovascular dysfunction in some individuals who experience severe intermittent hypoxia is also evident, and studies to determine the reasons for this absence would be of interest. Furthermore, equal emphasis on other physiological mechanisms (e.g., arousal and intrathoracic pressure swings) that have an equal or greater effect on cardiovascular function will serve to move the field forward (Mateika et al., 2015).

The most commonly used rodent model of OSA is the induction of intermittent hypoxia/reoxygenation by the rapid delivery of a hypoxic gas mixture to an airtight chamber, followed by flushing of the chamber with room air (e.g., 30s of hypoxia alternating with 30 s of normoxia). Data from both animal and human studies support mechanistic links between IH and its adverse impact at the tissue level. IH promotes oxidative stress by increased production of ROS and angiogenesis, increased sympathetic activation with blood pressure elevation, and systemic and vascular inflammation with endothelial dysfunction. These effects contribute to diverse multiorgan chronic morbidity and mortality affecting cardiovascular disease and metabolic dysfunction (Ayas et al., 2014; Dewan et al., 2015). Therefore, the use of cell or animal models to discriminate the acute effects induced by hypoxia from the adaptive mechanisms in chronic hypoxic contexts is relevant to the study of new pharmacological targets.

3.2.1.4. Endothelial dysfunction. Endothelial function has a primordial role in vasodilatation. Endothelium-derived vasorelaxing factors such as nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor (EDHF) are the main pathways for appropriate vasodilatation in most, if not all, vascular beds (Bernatova, 2014). Endothelial dysfunction comprises a lack of endothelial-derived vasodilatation and/or activation of endothelial-derived constricting factor. There are several degrees and origins of endothelial dysfunction, but undoubtedly hypoxia and oxidative stress are the most important causes of this condition (Salisbury & Bronas, 2015). Several years ago, it was proposed that eukaryotic cells live under a constant “oxygen paradox,” as they cannot survive without oxygen. Yet oxygen radicals and metabolites are dangerous to their existence (Davies, 1995). In fact, organisms can tolerate between 2000 and 4000 μM of O₂ concentration in tissues and about 8000 μM in alveoli (Decker & van Holde, 2011), ranging from 10 to 100 mmHg of pO₂ (Clanton, 1985). Lower or higher levels out of this range will induce a marked alterations in oxygen metabolism alteration with excessive O₂^{•−} production from several sources (Clanton, 1985). The most common sources of ROS are mitochondrial respiration, NADPH oxidase, XO at vascular level, which ultimately contribute to developing oxidative stress and endothelial dysfunction (Salisbury & Bronas, 2015). For instance, eNOS, the main source of NO in the endothelium uses O₂ as a substrate for NO. Therefore, hypoxia may decrease NO synthesis. Moreover, ROS also impairs NO availability by oxidizing tetrahydrobiopterin (BH₄) to dihydrobiopterin (BH₂), an essential co-factor for eNOS activity, as it stabilizes and activates the enzyme (Salisbury & Bronas, 2015). The lack of BH₄ leads to eNOS uncoupling resulting in O₂^{•−} rather than NO production. This vicious cycle increases ROS and further potentiates eNOS uncoupling. In addition, O₂^{•−} and NO quickly associate at a rate of $2 \times 10^{10} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ to peroxynitrite (ONOO[−]), further decreasing NO bioavailability and vasodilatation (Kissner et al., 1998). Conversely, ONOO[−] may further oxidize BH₄, inhibit guanylate cyclase, increase endogenous eNOS inhibitor ADMA, and activate PLA₂ and thromboxane release, thus promoting inflammation and vasoconstriction (Salisbury & Bronas, 2015; Siti et al., 2015).

Prostacyclin (PGI₂) is a prostanoid vasodilator derived from arachidonic acid (AA) and COX metabolism. Through the activation of its vascular prostacyclin receptor (IP), it enhances cyclic adenosine monophosphate (cAMP) intracellular levels and activates PKA, resulting in a decrease of [Ca²⁺]_i and vasorelaxation (Kawahara et al., 2015). Furthermore, PGI₂ inhibits vascular cell adhesion, thrombosis, inflammation, apoptosis, and proliferation (Bernatova, 2014). ROS was shown to decrease PGI₂ synthesis (Bachschmid et al., 2013) and shift the prostanoid balance promoting vasoconstriction. Finally, the endothelium-derived vasoconstrictor endothelin (ET-1) may increase ROS formation by activating NADPH, thus favoring OS (Siti et al., 2015).

All of the above, if prolonged, results in endothelial dysfunction. In fact, endothelial dysfunction is largely due to an increased production of ROS and decreased scavenging or antioxidant capacity, leading to reduced NO bioavailability and PGI₂ synthesis (Munzel et al., 2008).

3.2.1.5. Ex vivo models for cardiovascular assessment. Animal models are vital to understand the physiological and pathophysiological mechanisms of the cardiovascular system under hypoxia. *Ex vivo* models are critical to complement *in vivo* results with functional data. Nonetheless, “classic” physiology methods such as the Langendorff system and vascular myography are still valuable. They yield important data to elucidate hypoxic pathophysiology, for instance, cellular mechanisms of ischemic damage and regeneration (Webster et al., 2012), mediator regulation (Ahnstedt et al., 2012), and vasoactivity (Hasenau et al., 2011). However, in most cases, *in vivo* models for therapeutic target verification are irreplaceable since such models can closely simulate physiological conditions to pharmacological interventions (Vidavalur et al., 2008). There are several models of systemic hypoxia (Nakamura et al., 2011) and ischemic models of different organs or vascular beds. Of course,

these models differ in their technical complexity and, thus, in their feasibility.

Over many decades, the isolated retrograde-perfused heart preparation, better known as Langendorff, and the working heart have resulted in fundamental discoveries that form underpin of our current understanding of the biology and physiology of the heart. Both these experimental methodologies are invaluable in studying pharmacological effects on myocardial function and metabolism. In addition, they are also important for investigating of clinically relevant disease states such as IR injury, diabetes, obesity, and heart failure. With the advent of the genomics era, the isolated mouse heart preparation has gained prominence as an *ex vivo* research tool in the areas of gene modification and cardiac function.

Depending on the specific experimental conditions, the procedure might include the Langendorff or working heart model, constant perfusion or constant flow, and isometric force or isovolumetric pressure. Each of these experimental conditions has its own advantages and limitations that must be taken into account in the experimental design. Since the size, fragility, and high frequency of the murine heart pose additional challenges to researchers, this technique should be set up by experienced scientists.

The advantage of constant flow perfusion (due to the high fidelity of the peristaltic pump) is that precise and reproducible low degrees of flow can be induced to study the effect of low-flow ischemia in the heart (Assayag et al., 1998). Constant flow is also particularly well suited for studying the effect of vasoactive substances on coronary vasomotor tone. Thus, coronary pressure is a sensitive parameter that can be easily monitored, while coronary vascular resistance (an index of coronary vascular tone) is derived from this measurement using Ohm's law (Dijkman et al., 1997). It is possible and preferable to construct a Langendorff apparatus that incorporates elements of both, constant pressure and flow perfusion, thus, providing greater flexibility to the experimental design and even allowing the operator to readily switch between these two modes within a single experimental protocol.

Contractile force can be measured in the Langendorff-perfused mouse heart by attaching a hook with a suture through the apex of the heart and fixing the other end of the suture to an isometric force transducer, as originally described by Langendorff (Langendorff, 1895; Liao et al., 2012). Thus, tension is applied to the suture, and changes in contractions along the longitudinal axis of the heart can be measured. A preload can be applied through a traction device, and length-tension curves can be obtained by incremental increases of traction.

3.2.1.6. Vessel myography. In the mid-twentieth century, isolated vessels became a major interest in vascular research. Since then, many methods using pharmacological approaches have been successfully developed in *ex vivo* models, such as vascular slices or strips (Lawton, 1954, 1955; Leonard, 1957; Watton et al., 2009; Valdez-Jasso et al., 2011; Humphrey & Holzapfel, 2012), vascular explants (White, 1955; Meyrick et al., 1987; Rapraeger et al., 2013), and wire and pressure myography (Mulvany & Halpern, 1976; Spiers & Padmanabhan, 2005). Wire and pressure myography were vital for developing pharmacological studies in isolated vessels and discovering the key modulators of blood flow and local perfusion in many organs. In fact, wire myography was responsible for the discovery of locally controlled vasodilator/vasoconstrictor molecules such as NO, PGI₂, and ET-1, among others (Dusting et al., 1977; Furchgott & Zawadzki, 1980; Palmer et al., 1987; Masaki, 1989; Lerman et al., 1990). The isolated vessels can be studied under controlled experimental conditions, for example, under normoxia, hypoxia, and with or without Ca²⁺. In addition, the *ex vivo* conditions can be setup to reproduce those *in vivo* allowing the assessment of vessel function without nervous or humoral control. For example, elegant pharmacological studies have revealed the differential effects of chronic hypoxia in adapted and non-adapted animals (Herrera et al., 2007; Llanos et al., 2011; Moraga et al., 2011). The authors showed

that pulmonary arterial sensitivity of the contractile responses to nor-epinephrine was decreased in an adapted (llama) fetus relative to a non-adapted (sheep) one, whereas the relaxation sensitivity to NO was augmented (Llanos et al., 2011). Furthermore, vascular reactivity studies characterized pathophysiological changes in non-adapted individuals exposed to hypoxia, suggest an imbalance toward a vasoconstrictor state (Schindler et al., 2006; Herrera et al., 2007, 2008a,b). Furthermore, isolated vessels allowed the assessment of novel therapeutic proposals, such as the inhibition of phosphodiesterase V, a Ca²⁺ channel blockade or antioxidants (Herrera et al., 2008a,b, 2010; Fike et al., 2012; Giussani et al., 2012; Fike et al., 2013; Parrau et al., 2013), among many others. Hypoxic vasoconstriction may be altered by ROS through chronic hypoxia-induced mechanisms, such as vasoconstrictor prostanoids (Fike et al., 2011). In fact, it seems that ROS-reducing strategies may strongly improve vascular responses in chronic hypoxia-induced vascular dysfunction (Araneda & Tuesta, 2012; Giussani et al., 2012; Sylvester et al., 2012; Fike et al., 2013; Herrera et al., 2014a,c; Torres et al., 2015).

Conversely, in peripheral circulation, responses are markedly affected by chronic hypoxia that occurs with enhanced peripheral vasoconstriction, which is sensitive to α -adrenergic stimulation (Moraga et al., 2011). This effect was also shown in other lowland animals that exhibited an increased density of periarterial sympathetic nerve fibers in response to developmental chronic hypoxia (Ruijtenbeek et al., 2000). It is well known that many chronic diseases are associated with OS, such as obesity, hypertension, and diabetes. These conditions consistently involve peripheral vascular dysfunction, particularly with vasodilator impairment (Paravicini & Touyz, 2006; Marchesi et al., 2009; Hernanz et al., 2012; Tian & Chesler, 2012; Tian et al., 2012). Therefore, there is increasing clinical and scientific interest in antioxidant therapies that target the vasculature. In fact, several research groups have developed new therapeutic approaches in animal models and are testing the effects of antioxidants on vascular function.

Interestingly, many vascular responses and effects are common for both hypoxia and OS, supporting the idea that increased ROS generation occurs under hypoxia (Clanton, 1985; Guzy & Schumacker, 2006; Sylvester et al., 2012). Thus, transcription factors, such as Hypoxia-inducible factor (HIF)-1 α , nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and nuclear factor kappa B (NF- κ B), are activated by both stressors. These factors increase the transcription of vascular determinants such as ET-1, vascular endothelial growth factor (VEGF), and eNOS. In addition, some of the genes that modulate vascular tone are suggested to be epigenetically regulated by prenatal hypoxia (Kato et al., 2004; Fish et al., 2010; Nanduri et al., 2012; Giussani & Davidge, 2013), which leads to short- and long-term effects on the vasculature and its pharmacological responses. Many of the mechanisms underlying the vascular responses to hypoxia and OS, and the pharmacological characteristics of vessels have been discovered through vascular myography and are still being studied.

Clearly, wire and pressure myography have increased our ability to measure and interpret functions in an *ex vivo* representation of the *in vivo* condition. Since the isolated vessels are meticulously cleaned, and the effects of blood flow and content or autonomic nervous system control are removed, these systems allow the assessment of specific endothelium or smooth muscle cell function and pharmacological responses (Angus & Wright, 2000; Virdis & Taddei, 2011). In addition, the endothelium can be removed from the vessels (denudated), providing an option to determine intima or medial layer functions and their interactions (Furchgott & Zawadzki, 1980; Furchgott & Martin, 1985). Therefore, these methods are optimal *ex vivo* approaches for investigating the mechanisms underlying endothelial function and dysfunction. The integration of *in vivo* and *ex vivo* data will strengthen the experimental findings and provide conclusive answers in this area of research.

Examples of the mechanistic approaches of therapeutics agents used in cardiovascular models are shown in Table 1.

Table 1
Mechanistic approach of therapeutics agents used in pharmacological models associated with oxidative stress.

Approach	Treatment	Mechanism	Results	Ref []	
Femoral artery	Wire myography	Dexamethasone vitamins C and E	↓Vasoconstrictor pathways	Improve vascular dysfunction	Herrera et al. (2010)
	Wire myography	Vitamin C	NO-dependent and -independent mechanisms	Vitamin C restore vascular dysfunction and cardiac impairment induced by hypoxia	Giussani et al. (2012)
Middle cerebral artery	Wire myography	Melatonin	NO-independent mechanisms	Melatonin ↓ oxidative stress and improved vascular function	Herrera et al. (2014c)
Heart	<i>Ex vivo</i> IR (regional ischemia)	Achyranthes bidentata (Chinese herb)	↓ O ₂ [•] generation, MDA formation and ↓SOD activity	↓IS, plasma CK and LDH in rats	Tie et al. (2013)
	<i>In vivo</i> IR (regional ischemia)	NAC and ALP	NAC and ALP ↓ free15-F ₂ -IsoP	NAC and ALP improved diastolic and systolic function and LVEF	Wang et al. (2013)
	<i>Ex vivo</i> IR (global ischemia)	Polyunsaturated fatty acids	↓ TBARS and ↑antioxidant enzymes (CAT, SOD and GSH-Px) activities	↓ IS and improve LVEF	Castillo et al. (2014)
	Stroke by CAO	Flavocoxid	Inhibition COX-2 and 5-lipoxygenase	↓ Lipid peroxidation and IS	Singh and Chopra (2014)
Central nervous system	Tg2576 AD transgenic mice	Proanthocyanadins metabolites	ROS scavenger	Improve cognitive function	Wang et al. (2012)
	Perinatal asphyxia <i>in vivo/ex vivo</i>	Nicotinamide 2–24 h after insult	Inhibitor PARP-1, restore NADH/NAD ⁺	Antioxidant anti-inflammatory effects	Morroni et al. (2013)
	Fetal hypoxia <i>in vivo</i> sheep	Allopurinol	Xanthine oxidase inhibitor	Reduces hippocampal brain damage	Kaandorp et al. (2014)
Testis		Vitamin C	↑ Glutathione reductase activity in testis and epididymis	↑ Sperm count	Farias et al. (2010)
	Intermittent hypoxia	Melatonin	Prevents oxidative damage of enzymes like SOD, CAT, and GSH-Px	Protective effect on reduction in sperm motility	Farias et al. (2012)
	Chronic and intermittent hypoxia	Ibuprofen	Decreases hypoxia-induced vasodilatation	Protective effects on seminal lipid peroxidation	Vargas et al. (2011)
Renal system	Remnant kidney	Olmesartan	Blood flow in peritubular capillaries	Protective effect on tubular structure	Manotham et al. (2004)
	Chronic hypoxia	Prolyl hydroxylases inhibitor (medium-term administration)	↑ VEGF and EPO	↓ renal fibrosis and ↑ renal function	Gunaratnam and Bonventre (2009)
	Intermittent hypoxia	Melatonin	Decrease lipid peroxidation	↑ Renal function	Farias et al. (2012)

CAO, cerebral arterial occlusion; AKI, acute kidney injury; Fe-NTA, ferric nitriloacetate; DiOHF, 3',4'-dihydroxyflavonol; NAC, N-acetylcysteine; ALP, allopurinol; COX-2, cyclooxygenase-2; PGI₂, prostacyclin; NO, nitric oxide; O₂[•], superoxide radical; MDA, malondialdehyde; SOD, superoxide dismutase; F₂-IsoP, F₂-isoprostanes; TBARS, thiobarbituric acid-reactive substances; CAT, catalase; GSH-Px, glutathione peroxidase; VEGF, vascular endothelial growth; Nrf2, nuclear factor E2-related factor 2; H₂O₂, hydrogen peroxide; SIRT1, sirtuin 1; IS, infarct size; CK, creatine kinase; LDH, lactate dehydrogenase; LVEF, left ventricular ejection fraction; LVP, left ventricular pressure.

3.2.1.7. Pharmacological approaches

3.2.1.7.1. Antioxidants. In recent decades, several antioxidant therapies have been proposed for cardiovascular diseases associated with OS. In general, the outcomes in experimental models are positive and beneficial. However, when translated to human clinical practice, most of them are ineffective or present major adverse effects. For instance, the use of antioxidants for pregnancy and perinatal complications such as preeclampsia, placental insufficiency, and preterm neonates has been relatively successful in *ex vivo* and/or *in vivo* animal models (Miller et al., 2012; Giussani & Davidge, 2013; Cindrova-Davies, 2014; Herrera et al., 2014a,b). However, clinical trials evaluating antioxidant vitamins C & E in pregnant women and preterm neonates proved to be largely unsuccessful, probably due to a delayed administration of the treatment and low antioxidant levels in target tissues (Buonocore & Groenendaal, 2007; Cindrova-Davies, 2014). In general, limited correlation between *in vitro* and *in vivo* effects of antioxidant compounds, especially polyphenols, is probably due to factors related to the intestinal absorption and consequently the bioavailability of these compounds, which vary considerably according to their chemical structure, bound glycoside type and biotransformation processes (Bohn, 2014). It is important to note that attributing a cardioprotective effect to antioxidants molecules solely due to their antioxidant properties is a limited vision. Clearly, this effect relies on several other health-promoting properties, where these molecules need to have anti-hypertensive, anti-inflammatory, or anti-microbial properties. They need to protect mitochondrial function, regulate apoptosis/cell proliferation, or be epigenetic modulators. All these effects of antioxidants act synergistically to promote cardioprotection (Hollman et al., 2011).

The use of antioxidants as treatment for cardiovascular diseases during adulthood has given inconsistent results (Ozkanlar & Akcay, 2012;

Xu et al., 2014). For instance, although pharmaceutical approaches with vitamins A, C, & E were effective in preventing atherosclerosis in animal models, they have not given similar results in humans yet (Ozkanlar & Akcay, 2012; Riccioni et al., 2012; Siti et al., 2015). However, the use of antioxidants as a preventive strategy seems to have more convergent findings (Xu et al., 2014), suggesting that maybe exogenous antioxidants are only effective before the onset of OS. Antioxidants such as vitamin C, vitamin E, N-acetyl-L-cysteine, and resveratrol were shown to attenuate cardiovascular complications. In patients with existing cardiovascular disease (CVD), it is becoming evident that antioxidants may not be able to reverse the pathological changes in cardiac function. Nonetheless, the combined use of vitamin C, vitamin E, and NAC has been recommended for reducing side effects and to produce synergistic beneficial effects in CVD. Since resveratrol is an antioxidant and reduces blood cholesterol, it is expected that this agent would prevent and retard the development of atherosclerosis and coronary heart disease. In view of the inconsistent results showing an association between antioxidants and CVD, additional work is required to determine whether long-term antioxidant supplements exert beneficial effects in individuals with unhealthy lifestyle habits. It is also evident that vitamin C, NAC, and resveratrol are more effective than vitamin E on cardio protection (Baur & Sinclair, 2006; Chen et al., 2009; Zghonda et al., 2011; Ku et al., 2012; Marchal et al., 2012; Matos et al., 2012; Sabe et al., 2014; Xu et al., 2014).

Despite these controversies, the usefulness of antioxidants and their beneficial cardioprotective effects are supported by several studies, and hence, further investigation (Rodrigo et al., 2013). Interestingly, several of the above studies agreed that a diet rich in antioxidants, based on fruits and vegetables may be beneficial in the preventing cardiovascular events (Hertog et al., 1997; Genkinger et al., 2004).

Melatonin is a neurohormone with direct and indirect antioxidant actions. It is a strong scavenger, with increases the expression of some antioxidant enzymes and/or their activities and negatively modulates $O_2^{\cdot -}$ production from pro-oxidant processes (Hengstler & Bolt, 2007; Reiter et al., 2007). Statins are inhibitors of the cholesterol synthesis with demonstrated pleiotropic effects, such as antioxidant and anti-inflammatory capacities (Kotyla, 2014). In fact, studies have shown their ability to recover and protect endothelial function (Hermida & Balligand, 2014; Li & Forstermann, 2014), to improve eNOS coupling, and to decrease NADPH oxidase expression and activation (Schramm et al., 2012).

3.2.1.8. Polyunsaturated fatty acids. Based on results from cellular and molecular studies, the cardioprotection offered by omega-3 (Ω 3) fatty acids supplementation appears to be a combination of multiple effects that result in the improvement of cardiac hemodynamic factors, such as blood pressure, left ventricular diastolic filling, heart rate, and endothelial function (Mozaffarian & Willett, 2007). Recent studies showed that perfusion with Ω 3 fatty acids reduces infarct size and improves hemodynamic parameters in an isolated heart model (Richard et al., 2014). In a previous study from our group using global ischemia, we showed that severe IR induced tachyarrhythmias, an effect that was attenuated with Ω 3 fatty acids treatment (Castillo et al., 2014). In contrast, other studies found no differences in the left ventricular function after IR with PUFA supplementation, despite infarct size reduction (Abdukeyum et al., 2008).

The antioxidant effects of Ω 3 fatty acids are mainly related to its incorporation into the cell membrane and the modulation of antioxidant signaling pathways. Fish oil supplementation increases the expression and activity of the antioxidant enzyme SOD and decreases thiobarbituric acid-reactive substances (TBARS) in rats (Erdogan et al., 2004). Oxidized Ω 3 fatty acids react directly with the negative regulator of Nrf2, Keap1, initiating Keap1 dissociation with Cullin3, thereby inducing Nrf2-dependent antioxidant gene expression (Gao et al., 2007). This Ω 3-antioxidant reinforcement is associated with a reduction in the susceptibility of myocytes to ROS-induced IR injury, and to an increase in SOD and GSH-Px expressions and protein levels (Jahangiri et al., 2006). Animal studies showed that the cardioprotective effects of Ω 3 can be exerted through the upregulation of heat shock protein 72, a key preconditioning protein, and a dramatic increase in the Ω 3 content of myocardial membranes, which appears to facilitate a shift in oxidant IR injury (McGuinness et al., 2006). Also, Ω 3 may protect against acute lung injury induced by intestinal IR via the AMPK/SIRT1 pathway (Jing et al., 2014) and is associated to reducing macrophage infiltration, suppressing inflammation, inhibiting lung apoptosis, and improving the lung endothelial barrier.

3.3. Central nervous system

3.3.1. Models of stroke

Major advances over recent decades have been made in the prevention, treatment, and rehabilitation of ischemic brain disease; however, numerous challenges remain in translating new therapeutic approaches from the bench to the bedside.

Stroke can be classified into 2 categories: hemorrhagic and ischemic; the latter is the most prevalent form, accounting for up to 87% of all cases and is the target of most drug trials (Rosamond et al., 2007). A significant proportion of ischemic stroke is caused by atherothrombosis, which is defined as atherosclerotic plaque disruption with superimposed thrombosis. Stroke impacts overall antioxidant capacity and ROS production (Chang et al., 2005). Several studies have described increased levels of lipid peroxidation biomarkers, such as TBARS (Nanetti et al., 2011) and malondialdehyde (MDA) (Demirkaya et al., 2001), in the plasma and erythrocytes of stroke patients. The plasma levels of various non-enzymatic endogenous antioxidants, such as vitamin C (Cherubini et al., 2000), vitamin E (Chang et al., 1998), and the

antioxidant enzymes CAT and GSH-Px (Alexandrova et al., 2004), were also reported to be lower in ischemic stroke patients than in healthy control populations (Vakili et al., 2014). These data suggest that the aforementioned antioxidants are oxidized during the cerebral ischemia-reperfusion event. These OS biomarkers also seem to be related to the stroke outcome.

Experimental animal models are crucial to understand the mechanisms of neuronal survival following ischemic brain injury and to develop therapeutic interventions. Current studies on experimental stroke therapies are evaluating the efficiency of neuroprotective agents and cell-based approaches, primarily using rodent models of permanent or transient focal cerebral ischemia. In parallel, advancements in imaging techniques are improving mapping of the spatiotemporal evolution of the injury cortex and its functional responses (Canazza et al., 2014). Since surgical methods of middle cerebral artery occlusion (CAO) were first described in the 1970s and refined in the 1980s (Coyle, 1982), a number of additional techniques have been proposed, some of which avoid craniotomy. For instance, the intra-luminal suture model (Howells et al., 2010; Liu & McCullough, 2011) is commonly used in a large proportion of neuroprotection experiments. Other methods include the thromboembolic model (Sharkey et al., 1993), the coagulation or ligation model (Durukan & Tatlisumak, 2007), the endothelin-1 model (Bacigaluppi et al., 2010), and the distal artery compression model (Morancho et al., 2012).

3.3.2. Brain injury induced by endothelin-1

This model achieves reversible occlusion of the MCA through the application of the potent vasoconstrictor endothelin-1 (Soylu et al., 2012). It can be well reproduced in rats, offering benefits including the absence of lesions at the injection site and low mortality even in older animals, which are relevant considerations for stroke epidemiology in humans. Additional advantages include low invasiveness and the ability to both facilitate anatomical targeting of the lesion and implement in the absence of anesthetic agents (Howells et al., 2010), some of which may attenuate stroke-induced morbidity in small animal models. Endothelin-1 administration in rats induces OS and reduces antioxidant capacity probably through angiotensin II-dependent pathways (Ortiz et al., 2001). Some pharmacological interventions were used to abrogate this type of injury. Recently, in mice subjected to intermittent hypoxic protocols, cerebrovascular dysfunction was associated with OS and was attenuated using free radical scavenging or NADPH oxidase inhibition, for example, by using mice lacking the NOX2 subunit of NADPH oxidase (Capone et al., 2012).

3.3.3. Intermittent hypoxia and CNS

A number of mechanisms are associated with the effects of IH on neurocognitive processes. An early notion asserted that episodes of hypoxia could trigger apoptosis pathways in neurons in areas including the cortex and hippocampus, and could lead to morphological disorganization (Gozal et al., 2001; Goldbart et al., 2003). In fact, apoptosis in the hippocampal CA1 region in the rats exposed to IH, precedes the appearance of memory alterations (Gozal et al., 2001). However, effects on early phase (up to 1 h) and late phase (longer than 3 h) long-term potentiation were not addressed in this study.

To date, a key research question has been whether the degree of apoptosis is associated with cognitive impairment in animal models of IH. In fact, it is possible that chronic intermittent hypoxia can compromise the function of the neurons before apoptosis induction. Based on this view, IH can cause detrimental oxidative phosphorylation and consequently impair in the maintenance of neuronal ion gradients of neurons. Moreover, there were only a few attempts to examine the direct effects of chronic IH on the excitability of hippocampal neurons, and their synaptic transmission, in animal models of IH, such as obstructive sleep apnea. Nevertheless, it was shown that in the developing nervous system, IH will affect neuronal excitability and its maturation by altering

the expression of Na channels and others ion transporters (Gu & Haddad, 2003; Zhao et al., 2005).

The probable explanations for the detriment in neuronal function and/or apoptosis in the hippocampus and other brain areas are still lacking. However, it is highly probable that OS plays a significant role in neuronal damage due to IH induction (Wang et al., 2010). In this context, it is well established that there is increased expression of oxidative stress markers found in the brains of rats exposed to IH (Row et al., 2003; Zhan et al., 2005). The administration of antioxidants or over-expressing superoxide dismutase (Xu et al., 2004) attenuated ROS production and apoptosis in chronic IH-exposed animals. Recent evidence supports the role of NADPH oxidase and pro-oxidant imbalance in IH-induced oxidative stress (Nair et al., 2011). In this research, the mice model present significantly elevated levels of NADPH oxidase expression and activity, as well as MDA and 8-OHdG in cortical and hippocampal neurons. However, the source and the mechanism of ROS generation and its impact on neurocognitive deficits in IH are not yet clear.

3.3.4. Pharmacological approaches

The potential use of unconventional therapies, such as antioxidant defense system enhancement, might play a key role in the management of hypoxic stroke or pathophysiology-related states with OS occurrence. A combined therapy aimed to diminish brain injury and improve functional and structural outcomes is suggested. This therapy should be based on the time course of ROS production, the inhibition of ROS-producing enzymes, and the increased non-enzymatic antioxidant delivery to neurons (Wang et al., 2006). Xanthine oxidase activation is an important source of ROS early in the time course of ischemic stroke (Muralikrishna Adibhatla & Hatcher, 2006). Therefore, XO inhibitors, such as allopurinol, could be beneficial in mitigating initial ROS damage. This ROS generation together with ischemia leads to blood–brain barrier disruption, edema, and inflammation, all causing neutrophil infiltration to the infarct zone. In turn, further ROS generation due to the iNOS activation occurs. iNOS inhibitors such as vitamin C or L-nitroarginine could be used at this level (Harrison & May, 2009). Since NADPH oxidase is the main molecule responsible for ROS generation during hypoxic induction, vitamin C, vitamin E, or apocynin could be used in order to inhibit this enzyme (Weston et al., 2013). In addition, ROS scavengers such as resveratrol or N-acetylcysteine could be administered (Bastianetto et al., 2015).

Given this background, antioxidant defense system, enhancement constitutes a safe, low cost, and widely applicable therapy for hypoxic damage induced in the CNS. Taking into account the promising beneficial effects of this therapy, as well as its safety and low cost, it is emerging as a highly cost-effective alternative to pharmacological development in hypoxic models and neuronal damage.

Examples of mechanistic approaches of therapeutic agents used in central nervous system models are shown in Table 1.

3.4. Reproductive system

3.4.1. Hypoxic effects in females

Ovarian function depends on paracrine, endocrine, and neural factors, which interact to control follicular development and steroidogenesis. The development of ovarian follicles is an extremely dynamic process in which proliferation, differentiation, and death of somatic cells occur while the follicle passes through different stages of development. Finally, selected follicles release the oocyte (Ovulation) and are transformed in *corpora lutea*, while the others (not selected) die by follicular atresia (Oktem & Oktay, 2008). Follicles in different stages of development and the *corpora lutea* produce steroidal hormones that regulate the function of different tissues. Among the transcription factors involved in ovarian function, HIF is associated with follicular development and ovulation (Sirotkin, 2010). In fact, the hypoxic microenvironment produced locally in fast-growing *corpora lutea* induces HIF-1 α , which binds to the hypoxia response element region in the vascular

endothelial growth factor (VEGF) gene promoter in ovarian cells and induces upregulation of VEGF (Nishimura & Okuda, 2010). The vascularization of the *corpus luteum* and large antral follicles depends on VEGF and is necessary for the proper functioning of these ovarian structures, including the production of steroidal hormones such as progesterone. On the other hand, HIF-1 α expression can also be induced by the gonadotropin FSH and is related to the expression of key genes in follicular development such as LHR, VEGF, and inhibin- α (Alam et al., 2004). Once the *corpus luteum* is highly vascularized, it begins to be exposed to high levels of ROS production, which partially modulate the function and structure of the tissue. In fact, there are well-studied antioxidant enzyme activities that are fundamental to *corpora lutea* function (Al-Gubory et al., 2012). This point is important because abnormally high blood flow caused by increased vascularization of the *corpus luteum* could potentially lead to a local pro-oxidant status and impairing its function.

Furthermore, during pregnancy, trophoblast invasion and placental development depend on determinant levels of PO₂ and ROS (Jauniaux et al., 2006; Huppertz et al., 2014; Herrera et al., 2014b). In fact, one of the most challenging conditions in obstetrics is hypoxia and oxidative stress during development, both of which occur in high-altitude and low-altitude placental insufficiency. Hypoxia and oxidative stress may induce endothelial dysfunction, reducing the perfusion of the placenta and restricting fetal growth and development (Herrera et al., 2014b). Although many antioxidants have been proposed to treat complicated pregnancies, several have proved to be largely unsuccessful. However, further studies are needed to definitively determine the usefulness of antioxidants in such cases (Herrera et al., 2014b).

During the third trimester of pregnancy, placental growth reaches its limit and fetal oxygen demands markedly increase. This physiological condition decreases in terivous perfusion, generating local hypoxia and oxidative stress (Schneider, 2011; Redman et al., 2014). Therefore, for placental function, there is a greater chance of hypoxia and oxidative stress close to delivery (Herrera et al., 2014b). Several animal models have tried to replicate placental insufficiency, including carunclectomy, hyperthermia, umbilical ligation, umbilical occlusion, placental embolization, over/under nutrition, uterine artery ligation, and genetic knockouts in different species from rodents to large animals (Ergaz et al., 2005; Morrison, 2008). In addition, most of these models have exhibited hypoxia and oxidative stress. However, the relative contributions of these conditions and the mechanisms associated with the clinical evidence are still unknown and are currently under debate (Huppertz et al., 2014; Herrera et al., 2014b).

3.4.2. Chronic hypobaric hypoxia

Chronic hypobaric hypoxia has been associated with ovarian dysfunction and altered hormonal profiles in female rats (Macome et al., 1977; Martin & Costa, 1992). Chronic hypobaric hypoxia in rats is associated with decreasing fertility, polycystic ovaries, increasing estradiol biosynthesis, and altered estrous cyclicity (Martin & Costa, 1992). The appearance of cysts in the ovaries and the altered hormone profiles and estrous cyclicity demonstrate that hypoxia can affect follicular development and/or ovulation. However, the mechanisms explaining how oxygen availability modulates ovarian function were not assessed in these studies. In the field of domestic animal research, there is growing interest in studying the effects of hypobaric hypoxia on fertility because of the impact of altitude on the reproduction of animals bred for productive industry. For example, the exposure of ewes to a high-altitude hypoxic environment alters the morphology and function of their large antral follicles and *corpora lutea* (Parraguez et al., 2013). These animals exhibit smaller pre-ovulatory follicles and *corpora lutea*, with increased VEGF expression and luteal vascularization. The increased production of progesterone observed in the luteal phase of these animals could lead to negative feedback on gonadotropins that affect the maturation of follicles in the following cycle (Parraguez et al.,

2013). More studies are necessary to explain the mechanisms leading to ovarian dysfunction in animals exposed to a hypoxic environment.

Some therapeutic agents used against ovarian hypoxia, are shown in Table 1.

3.4.3. Intermittent hypobaric hypoxia

Hypobaric hypoxia notoriously affects any living organism. Since in high altitudes, the available environmental oxygen is reduced when the barometric or atmospheric pressure is lowered (exponentially descends in function of altitude), the lower partial pressure consequently leads to a decrease in the quantity of oxygen transported by the blood stream to all cells of the organism (Cummins & Taylor, 2005). According to a report issued in 2003 by the United Nation Environment Programme (UNEP), approximately 25% of the Earth's surface consists of mountains, while about 12% of the world's population lives in those regions (Programme UNE, 2003). In Chile, about 55,000 workers are subjected to an intermittent and chronic exposure to hypobaric hypoxia (over 3500 m asl). It is estimated that this population will grow as newly planned projects are due to commence. These projects will require miners other types of personnel are added such as border control staff, academics, tourists, and rural health officers (Richalet et al., 2002). Acute hypoxia occurs in individuals living at sea level who were exposed to high altitudes only for minutes, hours, or days to high altitude, as in the case of alpinists. Chronic hypoxia occurs in people who live permanently in high altitudes and occasionally get exposed to short periods (days) of normoxia (NM condition) as in the case of Peruvian or Bolivian residents. Finally, chronic intermittent hypoxia is the intermittent exposure to high altitudes periodically. An example of this is the life style of a section of the population in northern Chile and is usually associated to mining activity or military officers posted at the border (Abelson, 1976; Richalet et al., 2002).

3.4.4. Hypobaric hypoxia in animal models

Due to the complexity of performing studies on humans, one strategy is to study hypobaric hypoxia using animal models. Male Wistar rats used in such studies responded to this phenomenon with cardiovascular changes, which resemble the human response to conditions of high altitude (Fariás et al., 2005a,b). In such experiments on hypobaric hypoxia, the loss of corporal weight was a commonly observed phenomenon in the animal tested, compared to those living in normoxic conditions. According to Fariás et al. (2008), this variation loss of corporal weight is partly due to some degree of dehydration experienced by rats in high-altitude conditions. Apart from this loss of corporal weight of rats under hypobaric hypoxia, a variation in the weight of the organs was also observed. For example, in a study by Bustos-Obregón et al., where mice were exposed to Chronic Continuous Hypobaric Hypoxia, a significant reduction in the weight of the epididymis was observed between days 16 and 24 of the treatment in relation to the control group. This weight loss reverted after day 33, when the weight of the epididymis and "cauda" exceeded the control group (Bustos-Obregón et al., 2006). Studies on the testis have also revealed a significant weight loss in this organ both in Intermittent Continuous Hypobaric Hypoxia and Chronic Continuous Hypobaric Hypoxia conditions, being slightly higher in the latter (Fariás et al., 2005a,b).

3.4.5. Studies of hypobaric hypoxia on the male reproductive system

Hypobaric hypoxia is known to affect male fertility. The effects of hypoxia on fertility in both animals and human populations have been previously described (Abelson, 1976). For example, research on male rats has revealed significant differences in the histology of the testis compared to control animals in normoxia. When control rats were compared to either chronic or intermittent hypobaric and chronic continuous hypobaric hypoxia, a number of differences were observed: neovascularization, apoptosis, increase in scrotal temperature (Fariás et al., 2008; Liao et al., 2010), decrease in the testicular mass, increase in the interstitial space, reduction of the seminal epithelium, depletion

of cells in the epithelium, and vacuolization of epithelial cells. These changes further intensified following continuous treatment (Fariás et al., 2005a,b). Interestingly, some of these changes were observed in humans with varicocele, suggesting that they may have a similar etiology. Another study found comparable results in rat testicles subjected to similar conditions. However, here too the testes had differences in the amounts and form of spermatozoa present in the epididymis, which are affected by chronic exposure, leading to teratozoospermia (a situation in which 85% of the spermatozoa have abnormal morphology) and defective spermatozoa (Bustos-Obregón et al., 2006). However, this series of changes induced by chronic hypoxia are reversible in humans, provided that the subject exposed stays under normoxia conditions for a minimum period of 6 months. Thus, masculine fertility normalizes, with normal seminal count parameters, motility, form, and maturity of spermatozoa (Verratti et al., 2008). For these reasons, it was proposed that testicular hypoxia can affect fertility. Our findings on the effects of hypobaric hypoxia on male fertility, lead us to consider the specific activation of "Hypoxic hypobaric" pathways that in conjunction with other variables such as temperature, vascularization, and apoptosis, adversely impacts the fertility of individuals intermittently exposed to altitudes. Previous work demonstrated that people dwelling at sea level when exposed to altitudes of 7821 m above sea level had reductions in sperm counts and an increase in the number of dysmorphic spermatozoa (Okumura et al., 2003). Furthermore, exposure of monkeys and rats to hypoxia-induced spermatogenic arrest, degeneration of the germinal epithelium, and reduction in the volume of Leydig cells (Gosney, 1984; Biswas et al., 1985; Saxena, 1995). All these data suggest that when individuals native to normoxic and normobaric conditions are exposed to hypobaric hypoxia, spermatogenesis can be affected. However, it has not been possible verify whether hypoxia reduces fertility in humans (Vitzthum & Wiley, 2003). From a biomedical point of view, chronic intermittent hypoxia poses a potential health hazard to individuals who are periodically, routinely, and permanently exposed to it. An opinion survey among gynecologists in northern Chile indicated that 22.7% of participants state that they have found a relationship between infertility and working at high altitude, while 45.5% of them think that the infertility problem is related to shift work of mining centers (Fariás et al., 2013). Based on these results, we hypothesize that working environments may interfere with fertility and that intermittent exposure to hypoxia could also lead to health problems.

Recent tests reinforce the existence oxidative metabolism in rat epididymis subjected to hypobaric hypoxia due to increased expression of ROS regulatory enzymes (Fariás et al., 2010). This enhanced ROS production results in an increase in apoptosis at the germinal cell level, leading to a state of hypospermatogenesis (Turner & Lysiak, 2008). In a mature and fertile spermatozoon, the chromatin is highly compacted and stable, which protects against DNA damage (Sakkas et al., 1999). The preservation of chromatin integrity means the absence of single and double strand breaks in the DNA and no chemical modifications in its structure, which are indicators used for maintaining correct functioning of the spermatozoon (Shamsi et al., 2008). However, under oxidative stress, the sperm DNA becomes susceptible to ROS and oxidant radical attack thereby jeopardizing the integrity of the genetic material of the gamete, and therefore, masculine fertility (Griveau & Le Lannou, 1997). Similarly, under hypobaric hypoxia conditions, oxidative metabolism can lead to oxidative stress, which occurs when there is excessive production of ROS. This overcomes the antioxidant mechanisms of the cell, resulting in damage to proteins, lipids, and to DNA (Griveau & Le Lannou, 1997). Continuous and persistent oxidative damage to DNA can alter signaling cascades, and gene expressions induce or arrest transcription and cause errors in replication, resulting in genomic instability and mutations can lead to carcinogenesis, neurodegenerative disorders, infertility, and cardiovascular disease (Powell et al., 2005). Chronic or intermittent oxidative stress in spermatogenic cells, and consequently in the spermatozoa of workers subjected to high altitudes, is critical since it poses an imminent risk to the viability and quality of their

reproductive cells. However, despite the importance of understanding the effects of hypobaric hypoxia on the DNA of reproductive cells, there is a lack of studies, which associate hypobaric hypoxia, oxidative stress, and apoptosis to infertility. This is a key step in identifying pharmacological therapies to prevent male infertility. Therefore, under conditions of imminent damage to such cells, it is important to identify antioxidants that have a positive effect in the protection of germinal cells facing oxidative stress, which can then be incorporated into the diet of people exposed to high-altitude conditions (Farias et al., 2012). In other related pathologies with oxidative stress, such as heat stress or testicular torsion, ROS induces the activation of caspase 2 and/or calpains, which in turn activate the apoptotic pathway (Johnson et al., 2008; Jia et al., 2009; Lizama et al., 2009). The mechanisms involving constitutive (physiological) and externally induced apoptosis are far from understood. Germ cell death can be induced by hypobaric hypoxia and could lead to a decrease in sperm count. However, there is no information on the connection between oxidative stress and apoptosis in hypobaric hypoxia model. This is a key step in developing pharmacological therapies to prevent male infertility.

3.4.6. Pharmacological approaches

A variety of therapeutic approaches have been tested to prevent testicular effects in animals exposed to hypoxic conditions. The fact that ibuprofen appears to decrease oxidative stress has been attributed to its anti-vasodilatation activities in the testicle (Vargas et al., 2002). Furthermore, various antioxidant treatments can also partially reverse the effects of hypoxia in the testis, epididymis, and sperm (Reyes et al., 2012). However, the antioxidant melatonin gave contradictory outcomes (Farias et al., 2012). These outcomes might depend on the different routes of administration and the fact that this compound has both antioxidant and hormonal properties. Rats exposed to intermittent hypobaric hypoxia exhibited increased MDA content in the testicles and epididymides, which was reversed by the administration of the non-enzymatic antioxidants ascorbic acid and blueberry extract (Farias et al., 2010, 2012). The effects of hypoxia and oxidative stress on the male reproductive system are not as extensively studied as those on pregnant and non-pregnant females. Thus, they require additional studies to fully elucidate the consequences of intermittent and chronic hypoxia exposure. Some pharmacological approaches in this model are shown in Table 1.

3.5. Renal system

3.5.1. Role of hypoxia in kidney injury

Oxygenation of the kidney is characterized by a remarkable paradox. With regard to its weight, the kidneys are the very best perfused organs in an organism, receiving an overall oxygen supply of more than 80 mL/min for every 100 g weight, of which less than 10% is consumed for physiological processes (Legrand et al., 2011). Indeed, tissue oxygen tensions in the renal parenchyma are lower compared to most other organs, and much below those measured in the renal vein (Lubbers & Baumgartl, 1997). In fact, the renal medulla is considered a site with one of the lowest oxygen tensions in the body. The explanation for the discrepancy between high oxygen supply and low tissue oxygen tensions of the kidney lies in the unique architecture of the renal vasculature. In both the cortex and the medulla, branches of the renal arteries and veins run strictly parallel and are in close contact with each other over long distances. This parallel arrangement allows oxygen to diffuse from the arterial system into the venous system before it enters the capillary bed (Zhang & Edwards, 2002; Eckardt et al., 2005). Thus, oxygen is subjected to a countercurrent exchange comparable with urea, and this mechanism is particularly relevant in the renal medulla, where it leads to oxygen tensions below 10 mm Hg (Lubbers & Baumgartl, 1997).

The tubular segments of the kidney have very limited capacity for anaerobic energy generation and thus are dependent on oxygen to maintain active trans-tubular reabsorption of solutes, in particular

sodium. The combination of limited tissue oxygen supply and high oxygen demand is considered the main reason for the susceptibility of the kidney to acute ischemic injury (Bohle et al., 1996).

The impact of ischemia-related hypoxia on the progression of renal disease can be summarized using three main points. First, it has become clear that the peritubular capillary bed in the kidney, which provides the structural basis for adequate oxygen delivery to tubular cells, is a rather dynamic structure and that chronic diseases of the kidney are associated with a rapid decline in capillary density. Second, as a consequence of capillary loss and capillary hypoperfusion, tissue oxygen tensions usually decline in a diseased kidney (Schrier, 2010). Third, low oxygen tensions may not only impair energy generation, but also act as a regulator of cellular functions and as a specific stimulus for the induction of certain genes (Luo et al., 2014). Altogether, these findings suggest that hypoxia is an important factor in the progression of kidney disease.

3.5.2. Oxidative stress in acute kidney injury

Hypoxia plays a role in ischemic, toxicity-, and sepsis-induced acute kidney injury (AKI). Evolving hypoxia triggers renal adaptive responses that may mitigate the insult, leading to sub-lethal forms of cell injury. The unique capability of the kidney to downregulate oxygen consumption for tubular transport could represent one such adaptive response, which promotes maintenance of renal oxygenation, thereby preserving cellular integrity.

Homeostasis of renal parenchymal oxygenation is maintained by complex systems, which regulate and match regional blood perfusion and oxygen expenditure, principally for tubular transport. These systems are especially important in the renal outer medulla, where limited blood supply and oxygen delivery are barely sufficient for tubular transport demands and consequent oxygen needs (Heyman et al., 2012). Renal hypoxic stress may evolve from suppressed regional perfusion and increased oxygen utilization for tubular transport or their combination. Physiologically low medullary PO₂ leads to a particular vulnerability of this region to hypoxic injury, which predominantly involves S3 proximal tubules in experimental warm ischemia and reperfusion, and medullary thick ascending limbs in AKI models characterized by medullary hypoxia and continued tubular transport (Rosenberger et al., 2006). This difference in tubular susceptibility to injury reflects diverse cellular metabolism in different tubular segments. Proximal tubules are highly sensitive to hypoxia because they principally depend on oxidative catabolism. In contrast, distal tubular segments, especially mTALs, which are able to use glycolysis, endure severe hypoxia, provided that transport activity diminishes.

Cellular control of mitochondrial respiration is an additional potential adaptive response to hypoxic stress. ROS generation may also directly affect mitochondrial respiration and oxygen expenditure, while NO generated by eNOS normally competes with oxygen on cytochrome oxidase (Koivisto et al., 1999) and diminishes oxidative stress. Its production by iNOS is markedly upregulated during sepsis (Wu et al., 2007), leading to the formation of the highly toxic free radical peroxynitrite and ROS-mediated cell injury. Very little is known about cellular oxygen consumption in these settings, but it is believed that the dysregulation of mitochondrial respiration may develop, with relatively inefficient oxygen utilization and enhanced oxygen consumption, which may further enhance ROS formation. Thus, there appears to be a complex interplay between hypoxia signaling, cellular redox state, NO, and mitochondrial function, that likely has profound roles in renal oxygen regulation and dysregulation under normal circumstances and during hypoxic or septic AKI, respectively (Aksu et al., 2011).

3.5.3. Endotoxemia-related AKI

Since many of the inflammatory and hemodynamic consequences associated with severe sepsis are due to endotoxemia, preclinical experimental AKI studies have generally involved endotoxemic models (Wang et al., 2002). Endotoxin [lipopolysaccharide (LPS)] was been

shown to induce nitric oxide synthase (Schwartz & Blantz, 1999), enhancing arterial vasodilation. The latter activates the sympathetic and renin–angiotensin systems, which maintain blood pressure at the expense of renal vasoconstriction. The renal vasoconstriction predisposes to AKI, and protection has been demonstrated with renal denervation (Wang et al., 2002). Recently, a new model was proposed in which hemodynamic changes associated with increased oxygen consumption and ROS formation were observed (Singh & Li, 2012).

In an animal model of hyper-dynamic with sepsis state, this pathophysiological condition was associated with drop of the glomerular filtration rate. The only explanations that make physiological sense are a decrease in afferent arteriolar tone, with even greater decrease efferent arteriolar tone and intrarenal shunting (Calzavacca et al., 2014). In sepsis, if efferent vasodilatation is more pronounced than afferent vasodilatation, it could explain the rapid loss of glomerular filtration rate observed in clinical practice. A treatment that can increase efferent arteriole tone more than afferent arteriole tone would then be expected to restore GFR in sepsis. Endotoxin-induced acute renal failure could be caused by intrarenal ROS production, cytokine TNF- α /IL-1 β production, renal apoptotic cell death, and extrarenal activation of Toll-like receptor 4 (Cunningham et al., 2004; Guo et al., 2004). Indeed, The gp91phox-containing NAD(P)H oxidase was reported to be pivotal in LPS-induced TNF- α expression (Peng et al., 2005). ROS formation usually decreases during hypoxia (de Groot & Littauer, 1989). However, Nakanishi et al. (1995) showed that in the kidneys of rats exposed to hypobaric hypoxia for 21 days, SOD and catalase expression were unchanged, but levels of the lipid peroxidation marker malondialdehyde were significantly higher than in control rats. Some studies of the role of ROS during endotoxemia were undertaken by examining the effect of an exogenous antioxidant (MnTE-2-PyP) in endotoxemia-related AKI. The antioxidant properties of this agent include scavenging O₂⁻, H₂O₂, and ONOO⁻ (Patel & Day, 1999). The administration of this antioxidant before LPS was associated with a highly significant improvement in both GFR and RBF during endotoxemia. Indeed, renal protection was demonstrated when the antioxidant was administered 6 h after LPS. Also, a significant decrease in mortality at 24 h was also observed when the antioxidant was administered. A probable mechanism of these protective effects could be an increase in NO, secondary to decreased scavenging by ROS, contributing to the beneficial effect of the antioxidant against endotoxin-induced renal vasoconstriction and AKI development (Wang et al., 2003). This pathophysiological response in renal tissue is associated with increased expression of TLR4, NF- κ B, and the pro-inflammatory cytokine TNF- α , which makes them susceptible to further inflammatory insult. This increased susceptibility to LPS can be prevented with selective antioxidants or NF κ B inhibitors, providing novel insights into the pathophysiology of endotoxemia-related AKI, and others clinical conditions related to renal hypoxia, such as hepatic cirrhosis (Shah et al., 2012; Salama et al., 2013).

Therefore, the experimental determination of markers of oxidative stress and antioxidant status, in this model of AKI, can reveal the precise time of redox imbalance, with mechanistic implications for effective antioxidant supplementation.

3.5.4. Renal chronic hypoxia

The renal chronic hypoxia hypothesis suggests that primary glomerular disease will restrict post-glomerular flow and injure downstream, peritubular capillaries. In turn, microvascular dysfunction will cause a hypoxic milieu, and trigger a fibrotic response and the development of fibrosis in tubule-Interstitial cells (Palm & Nordquist, 2011). The fibrotic response exacerbates hypoxia and fibrosis by affecting neighboring capillaries and nephrons, leading to a vicious cycle that ultimately results in organ failure. This hypothesis provides an explanation for the progressive nature of fibrosis and has recently been corroborated *in vitro*, as well as, *in vivo* by studies showing a correlation between peritubular capillary loss and glomerular and tubulo-Interstitial scarring (Kang et al., 2002). Taken together, the decline in renal pO₂ precedes matrix

accumulation, suggesting hypoxia to be a pivotal stimulus for fibrogenesis that initiates and promotes fibrotic progression.

Increased oxygen consumption is closely linked to increased oxidative stress, which increases mitochondrial oxygen usage and reduces tubular electrolyte transport efficiency, both contributing to increased total oxygen consumption. The terminal electron carrier of the mitochondrial chain, cytochrome c oxidase, is oxidized at normal PO₂ and is normally only reduced when O₂ delivery is insufficient (Balaban et al., 1980). In the kidney, however, cytochrome c oxidase is already partially reduced during normal physiology (Epstein et al., 1982). Reducing proximal tubular QO₂ does not alter the redox state, but treatment with loop diuretics, e.g., furosemide, will increase oxidation of cytochrome c oxidase (Epstein et al., 1982). In conclusion, medullary O₂ delivery barely matches physiological demands and results in relative hypoxia as part of normal renal physiology, possibly explaining why a decreased medullary concentrating ability is the most common defect in AKI and generally considered a good indication of progressive renal failure (Brezis et al., 1984). Compromised hypoxic gene response can result in arterial hypertension, impaired salt handling, fibrosis, and/or oxidative stress can result (Tanaka & Nangaku, 2010). Two weeks of chronic infusion of angiotensin II (Ang II) to rats using osmotic mini-pumps resulted in hypertension and elevated oxidative stress due to NADPH oxidase activation (Welch et al., 2003). Interestingly, these rats also presented kidney tissue hypoxia. Activation of the renin–angiotensin system, as a compensatory mechanism to maintain normal systemic blood pressure, is also a possible explanation for the cortical hypoxia observed in chronically salt depleted rats (Stillman et al., 1994). In addition, oxidative stress will impair renal O₂ sensing, as concluded from the lack of increased VEGF, HO-1, and erythropoietin in diabetic rat kidneys. This is further supported by similar observations in several hypertensive animal models (Cowley et al., 2015). In salt-sensitive rats, an excess of ROS production from the medullary thick ascending limbs of Henle associated with increased reabsorption of excess sodium and water are presented, contributing to hypertension.

3.5.5. Chronic intermittent hypoxia in CKD

Animal models suggest that chronic hypoxemia induces sympathetic nervous system outflow and subsequently increases vascular resistance (Dempsey et al., 2010). Chemoreflex-mediated increases in sympathetic activity also trigger a neurohormonal response with the activation of the renin–angiotensin–aldosterone system, driving hypertension. In addition to the impact on blood pressure homeostasis, aldosterone has been linked to fibrosis and renal damage. Greene et al. (1996) demonstrated that aldosterone was the etiologic agent in glomerular sclerosis in the remnant kidney model in rats, with subsequent studies showing that this process could be reversed with aldosterone blockade (Aldigier et al., 2005). Obstructive sleep apnea, a clinical model of IH, is independently associated with cardiovascular diseases, including hypertension, coronary heart disease, heart failure, and stroke (Shahar et al., 2001). Several mechanisms are proposed to explain this excess cardiovascular risk among individuals with obstructive sleep apnea, including hypoxemia-induced endothelial dysfunction, accelerated atherosclerosis, and altered cardiovascular hemodynamics. Each of these factors can have a deleterious impact on renal function. Patients with obstructive sleep apnea appear to have increased aldosterone, placing them at risk for glomerular sclerosis (Adeseun & Rosas, 2010).

Obstructive sleep apnea-mediated hypoxemia is thought to produce an ischemic-reperfusion injury, which is associated with ROS burst. ROS are generated through both NADPH and XO pathways. ROS are also associated with pro-inflammatory mediators. For instance, NF- κ B is up-regulated, initiating a cascade of events that leads to leukocyte recruitment. Cytokines tumor necrosis factor- α , interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 are also increased (Quercioli et al., 2010). In addition, increased platelet aggregation, insulin resistance, and metabolic dysregulation appear to be involved in endothelial activation and oxidative stress occurrence (Lim et al., 2009). These

factors are also implicated in the initiation and progression of kidney disease. Furthermore, sleep apnea also has a negative impact on endothelial function. Therefore, sleep apnea can be defined as a new cardiorenal risk factor (Ozkok et al., 2014).

3.5.6. Pharmacological approaches

Hypoxia is the final common pathway to the end-stage renal failure. Ischemia of the kidney is induced by the loss of peritubular capillaries in the tubule–interstitium in the late stage of renal disease. Accumulating evidence also suggests a crucial role for hypoxia in the tubule–interstitium before structural microvasculature damage in the corresponding region, emphasizing the pathogenic role of this condition from an early stage of kidney disease (Nangaku, 2006). Given this background, therapeutic approaches against this final common pathway should be effective in a broad range of renal diseases. Presently, the administration of erythropoietin to correct anemia and the blockade of the renin–angiotensin system to preserve peritubular capillary flow and reduce OS are key to the improving kidney oxygenation. Indeed, the HIF transcription factor at the center of many cellular hypoxic response pathways will be an attractive target for therapeutic manipulation.

In a remnant kidney model, treatment with the angiotensin receptor blocker, olmesartan, is associated with the restoration of blood flow in peritubular capillaries and an improvement in the kidney oxygenation (Futrakul et al., 2005). Although these improvements in kidney oxygenation by the inhibition of the renin–angiotensin system are multifactorial, one important mechanism is the dilation of efferent glomerular arterioles and a consequent increase in blood supply to the downstream tubule–interstitium. Inhibitors of the renin–angiotensin system also serve as antioxidants and should ameliorate uncoupling of mitochondrial respiration, leading to more efficient use of oxygen. Supporting the latter mechanism, the administration of an angiotensin receptor blocker increased PO₂ in the cortices of the SHR and reversed the inefficient use of O₂ for Na⁺ transport (Welch et al., 2003).

With regards to recent pharmacological interventions, HIF activation could retard the progression of CKD in the remnant kidney and diabetes rat models (Nangaku & Eckardt, 2007). There is also evidence that the activation of HIF signaling in renal epithelial cells is associated with the development of CKD and that HIF activation may promote renal fibrosis (Higgins et al., 2008; Kimura et al., 2008). Previously, it was found an abundance of the nuclear protein of HIF- α in CKD exhibited dynamic changes; it was transiently activated in the early stage of CKD and suppressed in the middle and end stages. It suggests that the therapeutic activation of HIF at different stages of CKD might have different effects on CKD progression. Recently, treatment using the prolyl hydroxylase domain (PHD) inhibitor, l-mimosine, that was initiated at an advanced stage of progression predominantly activated HIF-2 α , upregulated erythropoietin, and VEGF expression, preserved the peritubular capillary network, and ameliorated the progression of CKD. There is growing interest in the development of PHD inhibitors as therapeutic agents in CKD (Yu et al., 2012).

Polyphenols have been used as antioxidant enzyme modulators, metal chelators, and ROS scavengers to attenuate the structural and functional damage to the kidney (Rodrigo & Bosco, 2006). Some experimental approaches provided evidence that some polyphenols, such as quercetin, have a protective effect on hypoxic injury in tubular cells. In proximal tubules subjected to hypoxic injury, quercetin attenuates the lipid peroxidation byproduct formation (TBARS) and LDH release (Pietruck et al., 2003). Moreover, the mitochondrial protection, determined by the effects of polyphenols in hypoxic pathophysiological conditions, can play a role to maintain the tubular function (Funk & Schnellmann, 2013). An experimental observation indicates that mitochondrial dysfunction in kidney proximal tubules is a limiting factor for ion transportation, being the resistance of complex I against the hypoxic injury an indicator for the energetic efficiency, fundamental aspect of mitochondrial dysfunction pathophysiology during AKI (Feldkamp et al., 2004). In agreement with these studies, this effect was associated

with the increased activity of two antioxidant enzymes (CAT and GSH-Px), and an enhanced GSH/GSSG ratio in the kidneys of rats receiving red wine polyphenols (Roig et al., 1999). Additionally, lipid peroxidation and nitrotyrosine in the kidneys and liver, negatively correlated with the polyphenol concentration of the administered red wine (Rodrigo et al., 2005; Drel & Sybirna, 2010), an observation that partially corroborated the renoprotective effect previously reported for resveratrol (Bertelli et al., 2002; de Jesus Soares et al., 2007). In other AKI models related to the induction of hypoxic conditions, resveratrol exerts renoprotective effects via radical scavenging and antioxidant activities, which appear to involve the inhibition of tissue neutrophil infiltration (Sener et al., 2006; Kitada & Koya, 2013). Other antioxidants such as vitamin C (Ustundag et al., 2008), melatonin (Aydogdu et al., 2006), and N-acetylcysteine (NAC) (Polo-Romero et al., 2004; Zhu et al., 2007) have been successfully used for this type of renal injury. It is important to note that in most of these studies markers of renal injury and function are also evaluated, focusing on the clinical applicability of these models.

4. Concluding remarks

Models of hypoxic and oxidative stress injury at different stages of life and in different organs have revealed the complexity of several diseases related to hypoxia.

A large number of experimental studies have demonstrated that the pharmacological models of study in hypoxia are strongly dependent on the time of exposure and the method used to determine functional impairment. New protective strategies were found to be reproducible in preclinical studies, across a range of studies with *in vitro*, *ex vivo*, and *in vivo* experimental models. Nonetheless, new pharmacological strategies should be further tested with experimentally induced pathologies that mimic clinical settings (age, co-morbidities, and specific organ functions). Furthermore, combining drugs with different mechanisms of action should be considered as a strategy for the treatment of hypoxia and oxidative stress-related diseases to achieve additive or synergistic benefits. Moreover, once the drug is used in clinical trials the effectiveness of the strategy can be evaluated to confirm whether the intervention is truly efficacious.

Hypoxia and oxidative stress are silently damaging the cellular structures and functions. Unfortunately, the majority of diagnoses are performed too late, resulting in the failure of most pharmacological attempts to recover function. Preventive behaviors, early diagnosis, and additional treatments are needed to overcome hypoxia and oxidative stress in disease.

Conflict of interest

The authors declare no conflict of interest.

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