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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin reduces oxidative stress and improves vascular function in pulmonary hypertensive newborn sheep

Abstract: Pulmonary hypertension of the newborn (PHN) constitutes a critical condition with severe cardiovascular and neurological consequences. One of its main causes is hypoxia during gestation, and thus, it is a public health concern in populations living above 2500 m. Although some mechanisms are recognized, the pathophysiological facts that lead to PHN are not fully understood, which explains the lack of an effective treatment. Oxidative stress is one of the proposed mechanisms inducing pulmonary vascular dysfunction and PHN. Therefore, we assessed whether melatonin, a potent antioxidant, improves pulmonary vascular function. Twelve newborn sheep were gestated, born, and raised at 3600 meters. At 3 days old, lambs were catheterized and daily cardiovascular measurements were recorded. Lambs were divided into two groups, one received daily vehicle as control and another received daily melatonin (1 mg/kg/d), for 8 days. At 11 days old, lung tissue and small pulmonary arteries (SPA) were collected. Melatonin decreased pulmonary pressure and resistance for the first 3 days of treatment. Further, melatonin significantly improved the vasodilator function of SPA, enhancing the endothelial- and muscular-dependent pathways. This was associated with an enhanced nitric oxide-dependent and nitric oxide independent vasodilator components and with increased nitric oxide bioavailability in lung tissue. Further, melatonin reduced the pulmonary oxidative stress markers and increased enzymatic and nonenzymatic antioxidant capacity. Finally, these effects were associated with an increase of lumen diameter and a mild decrease in the wall of the pulmonary arteries. These outcomes support the use of melatonin as an adjuvant in the treatment for PHN.

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Key words: antioxidant system, chronic hypoxia, nitric oxide, oxidative stress, pulmonary hypoxic vasoconstriction, vascular function

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Introduction

More than 140 million people inhabit worldwide above 2500 m of altitude, a geographical condition that challenges pulmonary physiology in hypobaric hypoxia [1]. The chronic exposure to hypoxia at high altitude induces an increased vasoconstriction and cardiac afterload, deriving in elevated pulmonary arterial pressure and pathological remodeling [2, 3]. These changes are the key features of the pulmonary hypertension syndrome, a condition that may appear at any age but is more prevalent in newborns due to the major changes that take place in the pulmonary circulation at birth [4]. In fact, PHN is associated with high neonatal mortality and morbidity in low and high altitude [5, 6]. Moreover, children who survive may have long-lasting effects such as decreased postnatal growth and neurological, respiratory, and cardiac complications that often persist later in life [7–9]. When taking into account the increasing population living at high altitude and the environmental-associated perinatal pathologies, pulmonary hypertension of the newborn (PHN) is a current public health issue [6, 8]. However, the precise PHN prevalence at high altitude is not define, but several studies agree that being gestated and born above 2500 mt increases the chances of developing cardiopulmonary problems in the early postnatal period [3, 6, 8, 10]. The common features to PHN are excessive vasoconstriction, endothelial dysfunction, and pathologic remodeling of the pulmonary vascular bed [11–13]. These characteristics have been described and analyzed in experimental models and clinical evidence on newborns gestated in chronic hypoxic conditions showing similar patterns in the pulmonary circulation [6, 14–17]. Further, one of the potential mechanisms involved in the etiology of pulmonary hypertension is the *ROS hypothesis*, which suggests that reactive oxygen species (ROS) excess could be a main mediator of the hypoxic vascular constriction and endothelial dysfunction in the small pulmonary arteries [13, 18]. ROS are free radicals produced as by-products of oxidation–reduction (Redox) reactions. At low 'physiological' levels, ROS are known to act as important signaling molecules. However, in pathological conditions, ROS excessive production can overwhelm the cellular antioxidant capacity resulting in oxidative stress [19]. During hypoxia, an important source of ROS is complex III in the mitochondrial respiratory chain of the vascular smooth muscle cells [20]. Further, ROS can trigger the Ca^{2+} release from ryanodine-sensitive stores [18, 21], increase synthesis of cyclic adenosine

diphosphoribose (cADPR) [22], activate AMP kinase [23], and increase phosphodiesterase-5 (PDE5) activity [24] in pulmonary vascular smooth muscle cells. All of the above will determine a state of increased vasoconstriction. Moreover, ROS influence cellular processes in vascular remodeling by switching on signaling cascades such as extracellular signal-regulated kinases (ERKs), mitogen-activated protein kinases (MAPKs), receptor and nonreceptor tyrosin kinases, protein tyrosin phosphatases, and increasing deposition of extracellular matrix proteins [25]. Further, a dominant mechanism of endothelial dysfunction associated with oxidative stress is the nitric oxide inactivation by superoxide and consequent formation of peroxynitrite [26]. Therefore, oxidative stress has been proved as determinant in the development of pulmonary hypertension, and several antioxidant treatments have been proposed in perinatal medicine [27], where melatonin has a number of important beneficial properties relative to other antioxidants [28–32]. Melatonin features as an antioxidant include a direct scavenger action, the upregulation of antioxidant enzymes such as superoxide dismutase (SOD), peroxidases, and enzymes of glutathione supply and its ability to downregulate pro-oxidant enzymes such as lipoxygenases [28, 33–35]. In addition, melatonin has been considered as a mild vasodilator agent in the pulmonary bed [36, 37]. However, melatonin is not physiologically present in the circulation during the early neonatal period [38, 39] fact that may be favoring oxidative stress.

Currently, the treatment for PHN relies on inhaled NO, calcium channel blockers, and PDE5 inhibitors, which have a high rebound once the treatment is removed [40, 41]. Taking into account the multiple vascular and antioxidants effects of melatonin and its low adverse effects [42], we propose a postnatal treatment with melatonin to decreased oxidative stress and revert pulmonary hypertension in chronically hypoxic neonatal lambs. Therefore, we hypothesized that a daily oral dose of melatonin improves pulmonary vascular function, enhances the antioxidant capacity, and decreases oxidative stress in the neonatal lung of high-altitude pulmonary hypertensive lambs.

Materials and methods

All animal care, procedures, and experimentation were approved by the Ethics Committee of the Faculty of Medicine, University of Chile (protocol CBA N° 0398 FMUCH) and were conducted in accordance with the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Animals

Twelve newborn sheep (*Ovis aries*) were gestated, born, and studied at high altitude (Putre, 3600 m), randomly divided into two groups. The control group received vehicle (CN, n = 6, 1.4% ethanol 0.5 mL/kg) and the treated group received an oral dose of melatonin (MN, n = 6, melatonin 1 mg/kg in 1.4% ethanol 0.5 mL/kg), daily for 8 days (4 to 11 days old). To maintain melatonin normal endogenous rhythm, the daily dose was given at dusk (18:00). Animals were daily weighed and monitored during the experimental period.

Surgical preparation and in vivo experiments

All lambs were instrumented at 3 days old for daily hemodynamic and blood gases monitoring. In brief, at 3 days old, lambs were anaesthetized with a ketamine–xylazine association (10/0.04 mg/kg I.M.) with additional local infiltration of 2% lidocaine in the incision area. Polyvinyl catheters were placed in the descending aorta and inferior vena cava via hindlimb vessels. In addition, a Swan–Ganz catheter was placed in the pulmonary artery via right external jugular vein [16]. Pulmonary arterial pressure (PAP), systemic arterial pressure (SAP), and heart rate (HR) were recorded every morning (between 9:00–11:00 h for 30 min) with a data acquisition system (Powerlab/8SP System and Chart v4.1.2 Software; ADInstruments, Bella Vista, NSW, Australia) connected to a computer. In addition, cardiac output (CO) was determined in triplicate by the thermodilution method with the Swan–Ganz catheter connected to a cardiac output computer (COM-2 model; Baxter, Irvine, CA). Pulmonary vascular resistance (PVR) was calculated as described previously [16]. Daily arterial blood gases were analyzed, and venous blood samples were taken at days 1 and 8 of treatment. All in vivo measurements were performed in unanesthetized animals in a home-made metabolic bed.

Ex vivo and in vitro experiments

At the end of treatment, lambs underwent euthanasia with an overdose of sodium thiopentone (100 mg/kg, slow I.V. infusion). At dissection, lungs were weighed and pulmonary tissue was obtained for ex vivo and in vitro studies.

Pulmonary artery reactivity by wire myography

The right lung was dissected and immediately immersed in cold saline. Fourth to fifth-generation pulmonary arteries were dissected from the medial lobule of the right lung. Isolated arteries were mounted in a wire myograph, maintained at 37°C, and aerated with 95% O_2-5% CO_2. Cumulative concentration–response curves (CCRCs) were performed for potassium (K^+), serotonin (5Ht), methacholine (MetCh), and sodium nitroprusside (SNP). The vasodilator CCRCs were performed following preconstriction with 10^{-6} M of 5Ht. CCRCs to MetCh were repeated following endothelial nitric oxide synthase (eNOS) blockade with 10^{-5} M of L-NAME to assess the NO-dependent and independent components of the endothelial-derived vasodilatation [32, 43]. Vasoactive responses were analyzed by fitting experimental data to a Boltzmann equation or a dose–response sigmoidal function as appropriate (Prism 5.0; GraphPad Software, La Jolla, CA). Contractile responses were expressed in terms of wall tension or percentage of potassium maximal contraction (mN/mm or $\%K_{max}$) and relaxation responses as a percentage of 5Ht-induced contraction. Sensitivity was calculated as EC_{50} or pD_2, where $pD_2 = \log[EC_{50}]$, with EC_{50} being the concentration at which 50% of the maximal response was obtained [43].

NO_x analysis

Lung tissue NO-concentrations were determined using the Griess reaction by measuring combined oxidation products of NO (NO_x), nitrite (NO_2^-), and nitrate (NO_3^-) after reduction and final conversion to an azo compound in a commercial colorimetric assay, according to the manufacturer's instructions (Cayman Inc., Ann Arbor, MI) [44].

Transcript expression

Total RNA purification from lung tissue, cDNA synthesis, and PCR amplification was performed as described previously [45]. Primers were designed for the amplification of partial DNA sequences from eNOS (forward 5′-TGGCT GGTAGCGGAAGG-3′, reverse 5′-TCAGCTCGCCAAG GTGACCAT-3′); SOD2 (forward 5′-CGGTGG-TGGAG AACCCAAAGGG-3′, reverse 5′-CTGCAAGCTGTGTA TCGTGCAGTT-3′); CAT (forward 5′-AAATGGCTCAC TTTGACCGGGAGA-3′, reverse 5′-AATTTCA-CTGC AAAGCCACGAGGG-3′); GPx1 (forward 5′-CTGGTCG TGCTCGGCTTCCC-3′, reverse 5′-GGGTCG-GTCATG AGAGCAGTGG-3′), and 18S-rRNA (forward 5′-TCA AGAACGAAAGTCGGAGG-3′, reverse 5′-GGACATCT AAGGGCATCACA-3′, as housekeeping gene). The PCR products were visualized under ultraviolet light, digitally photographed, and quantified by densitometry (Scion Image Beta 4.02 Win; Scion Corp., Frederick, MD, USA).

Protein expression

Protein expression of eNOS, SOD2, CAT, GPx1, and β-actin was determined in total lung lysates by immunoblot with specific antibodies (anti-eNOS, 610296, BD Transduction Laboratories, Franklin Lakes, NJ, USA; anti-Mn-SOD, 06-984, Millipore, Billerica, MA, USA; anti-CAT, ab1877, Abcam Laboratories, Cambridge, UK; anti-GPx1, ab22604, Abcam Laboratories, Cambridge, UK; and anti-β-actin, AC-15, Thermo Fisher scientific, Waltham, MA, USA, respectively) as described elsewhere [46]. The signals obtained on immunoblot determinations were scanned and quantified by densitometric analysis with a chemiluminescence detection device (Odyssey Imaging System, Li-Cor Biosciences, Lincoln, NE, USA).

The detection of activating eNOS phosphorylation at residue (Ser 1177) was quantified in lung homogenates by enzyme-linked immunosorbent assay (PathScan® Phospho-eNOS Ser1177 Sandwich ELISA Kit #7980, Cell Signaling Technology, Inc. Danvers, MA, USA), following the manufacturer's instructions.

Oxidative stress markers

The oxidative stress markers NT and 4-HNE were also measured by Western blot with specific antibodies (anti-NT, Millipore, 05-233 and anti-4-HNE, Abcam Laboratories, ab46545). 8-Isoprostanes concentrations in plasma and lung homogenates were measured with a specific enzyme

Table 1. Arterial blood gases at the end of the treatment. pH, PCO_2, PO_2, hemoglobin concentration (Hb), Hemoglobin saturation by oxygen (SaO_2), and arterial blood oxygen content (O_2 cont) in newborn sheep gestated at high altitude (3600 m) during the treatment period with vehicle (control, CN) or with melatonin (MN). Values are means ± S.E.M.

	Control (CN)	Melatonin (MN)
pHa	7.483 ± 0.016	7.475 ± 0.010
PCO_2, mmHg	37.8 ± 2.0	35.1 ± 1.2
PO_2, mmHg	44.6 ± 2.4	46.8 ± 0.8
Hb, g/dL	12.88 ± 0.62	12.84 ± 0.40
SaO_2, %	70.8 ± 1.9	72.9 ± 3.6
O_2 cont, mL/dL	12.12 ± 0.64	12.46 ± 0.41

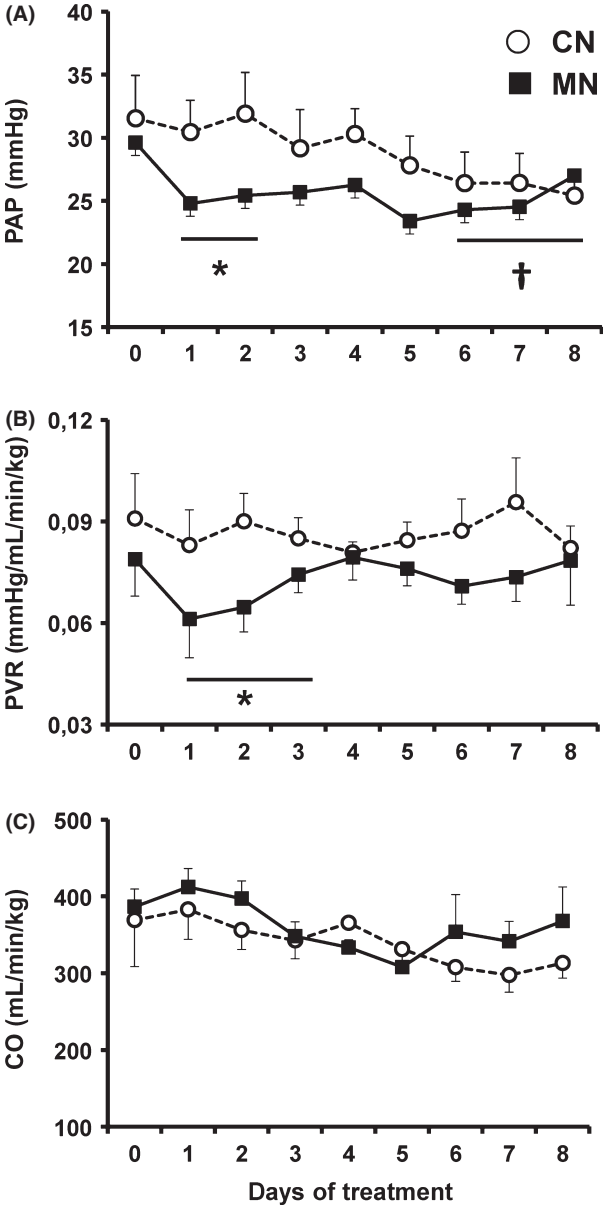

Fig. 1. Cardiopulmonary postnatal evolution. Pulmonary arterial pressure (PAP, A), pulmonary vascular resistance (PVR, B) and cardiac output (CO, C) in newborn sheep gestated at high altitude (3600 m) during the treatment with vehicle (CN, open circles) or with melatonin (MN, closed squares). Values are means ± S.E.M. Significant differences ($P \leq 0.05$): †versus. day 0,*versus CN at equivalent days.

immunoassay (EIA) kit following the manufacturer recommendations (Cayman Chemical, Ann Arbor, MI) [47].

Antioxidant enzyme activities

Activity of antioxidant enzymes in lung tissue homogenate was measured using the Superoxide Dismutase (SOD) Activity Assay Kit (K335-100, Biovision, Milpitas, CA, USA), OxiSelect Catalase Activity Assay Kit (STA-341, Cell Biolabs Inc., San Diego, CA, USA) and the Glutathione Peroxidase Assay Kit (703102, Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's guidelines. Sample protein concentration was used for normalization purposes [48].

FRAP

Total antioxidant capacity of the plasma was assessed by the ferric-reducing ability of plasma (FRAP), as previously described [49].

Plasma melatonin

Plasmatic melatonin concentration was assessed by RIA as previously described [50]. Samples were collected under sterile conditions into chilled EDTA tubes (2 mL K+/EDTA) and centrifuged, and plasma was kept in liquid nitrogen until analysis. Plasma samples were extracted with diethyl ether prior to assaying. The assay used melatonin antiserum (Guildhay Antisera Ltd., Guildford, Surrey, UK) and [O-methyl-3H]-labeled melatonin (85 Ci mmol/L; Amersham Biosciences AB, SE-751 84, Uppsala, Sweden) as a tracer.

Pulmonary morphostructural analyses

Left lung was perfused at 25 mmHg with saline and 4% paraformaldehyde (PFA). Afterward, 1 cm^3 blocks were immersed-fixed with 4% PFA for 24 h at 4°C, followed by washes and conservation in PBS + sodium azide 0.1% at 4°C. Fixed samples were embedded in paraffin and cut into 10-μm slides. Hematoxylin–Eosin and van Gieson staining were performed for vascular morphometry. Images were captured at 10× and 40× with a digital camera coupled to a microscope (Olympus BX-41, Olympus Corporation, Tokyo, Japan), and arterial dimensions were measured using an image analysis software (Image-Pro Plus 6.2; Media cybernetics, Inc., Rockville, MD, USA). The diameter and percentage of wall thickness were calculated as described previously [17, 51].

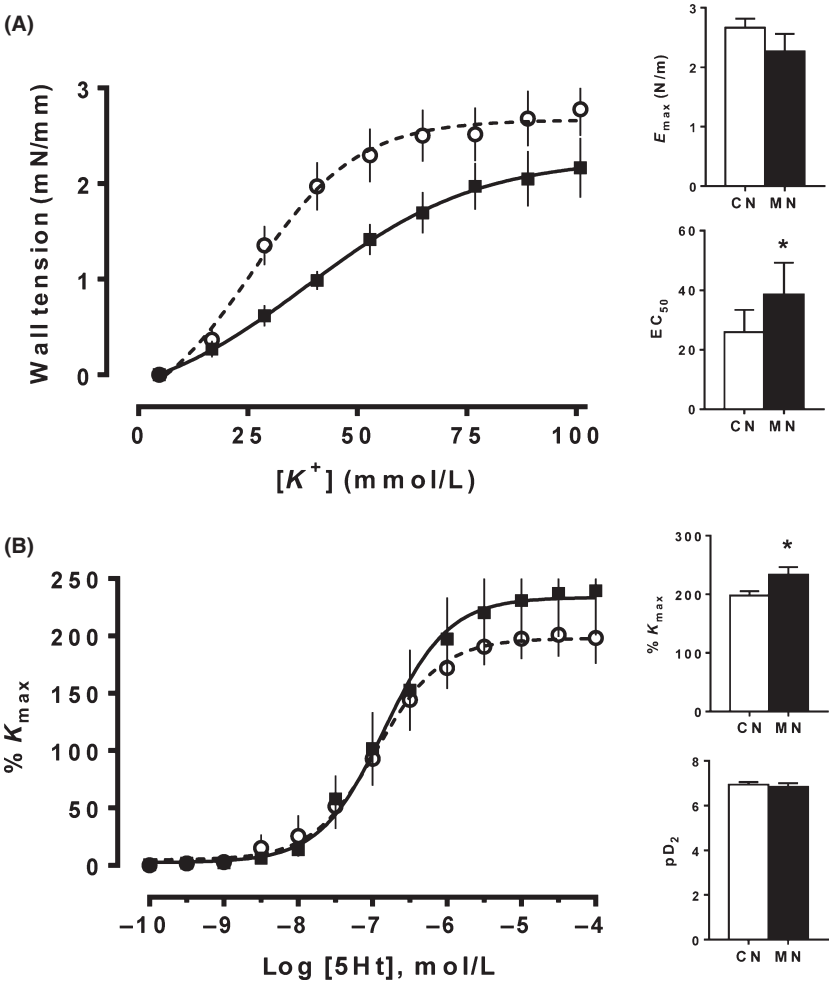

Fig. 2. Vasoconstrictor function of small pulmonary arteries. Values are the means ± S.E.M. for the vascular response to potassium (K^+, A) and to serotonin (5Ht, B). Maximal responses (E_{max} or K_{max}) and sensitivity (EC_{50} or pD_2) were calculated (inserted histogram). Groups are control (CN, open circles/bars) and melatonin-treated (MN, closed circles/bars) lambs. Significant differences ($P \leq 0.05$): *versus CN.

Statistical analyses

All data were expressed as means ± S.E.M. Shapiro–Wilk test was used to assess normality of the data. For the in vivo analyses, daily recordings were average. For the ex vivo analyses, the vascular response to potassium was analyzed using the Boltzmann sigmoidal analysis, and the maximal effective tension (E_{max}) and the half maximal effective concentration (EC50) were determined. All other CCRCs were analyzed using an agonist-response best-fit equation, where the maximal vasomotor response was expressed as the percentage of the submaximal contraction induced by K^+ [64 mM] or 5Ht [10^{-6} M]. The sensitivity was expressed as pD2 (−logEC50) [16, 43]. Differences in the vascular responses to methacholine were compared by calculating the area under the curve for estimating the NO-dependent and independent contribution as published elsewhere [43]. Ratios and percentages were arcsine-transformed prior to statistical analysis. All results were compared statistically by an unpaired t-test unless otherwise stated. Significant differences were accepted when $P \leq 0.05$ (Prism 5.0; GraphPad Software).

Results

At birth, all animals showed similar biometric variables as previously reported [32]. Further, blood gases were similar between groups at 3 days old (data not shown), and these values were preserved until the end of the experimental protocol (Table 1).

Cardiopulmonary variables

At surgery (3 days old, prior treatment), both groups of animals showed similar values in pulmonary arterial pressure (PAP), pulmonary vascular resistance (PVR), and cardiac output (CO) (Fig. 1). In the first and second days of treatment (5–6 days of age), MN showed an early significant decline in PAP and PVR, but reached similar levels in both groups by the end of treatment (Fig. 1A,B). In contrast, CO was maintained in similar levels in both groups throughout the experimental period (Fig. 1C). Along the daily recordings, there was no evidence of patent *ductus arteriosus*, effective closure that was confirmed at euthanasia in all animals.

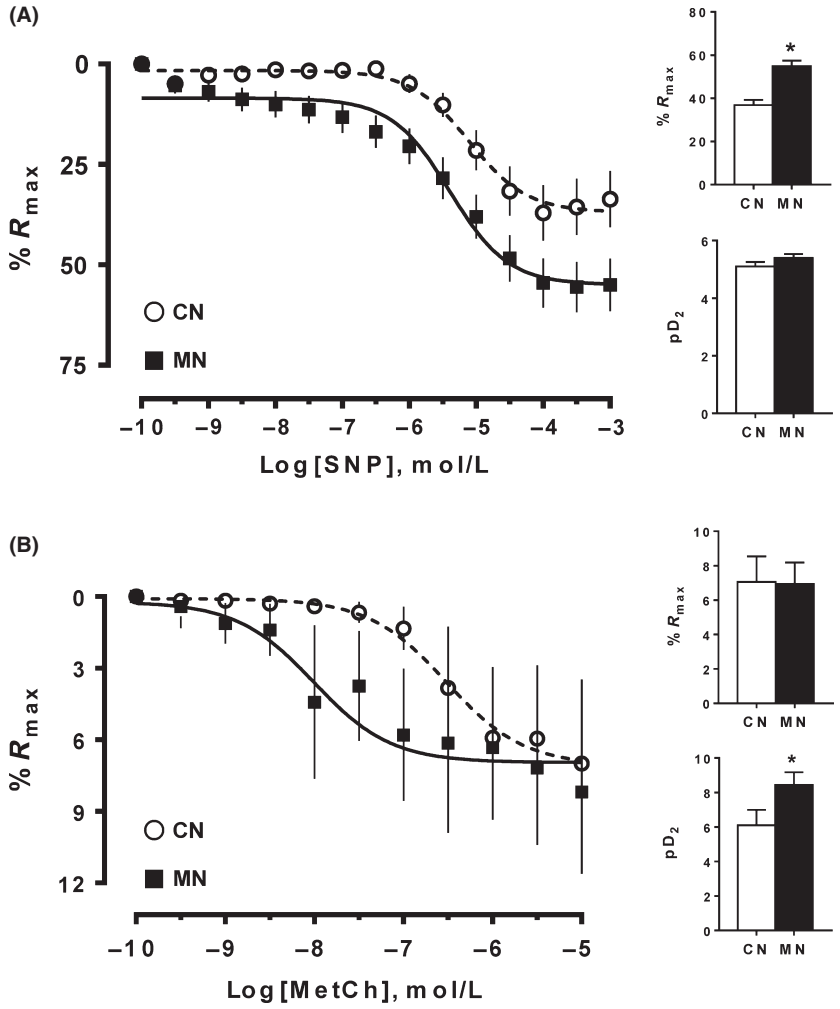

Fig. 3. Vasodilator function of small pulmonary arteries. Values are the means ± S.E.M. for the vascular response to sodium nitroprusside (SNP, A) and methacholine (MetCh, B). Maximal responses (%R_{max}) and sensitivity (pD_2) were calculated (inserted histogram). Groups are control (CN, open circles/bars) and melatonin-treated (MN, closed circles/bars) lambs. Significant differences ($P \leq 0.05$): *versus CN.

Small pulmonary arteries function

The contraction of small pulmonary arteries (SPA) to potassium showed a similar maximal response in both groups, but decreased sensitivity (EC50) in MN relative to CN (Fig. 2A). Further, serotonin induced a stronger vasoconstriction (%K_{max}) in MN relative to the control group, with similar sensitivity between groups (Fig. 2B).

We assessed vasodilator responses by exposing SPA rings to the nitric oxide donor, SNP, observing a higher maximal relaxation in MN than CN, with similar sensitivity between groups (Fig. 3A). In addition, the endothelial-derived vasorelaxation assessed by MetCh showed similar maximal vasodilator responses, but higher sensitivity in MN than CN lambs (Fig. 3B). When estimating the vasodilator components of the endothelial-derived relaxation, both the NO-dependent (MN 547 ± 120 versus CN 498 ± 99 AUC) and NO independent (MN 78.4 ± 17.1 versus CN 6.1 ± 1.2 AUC) dilatation were increased in MN relative to CN (Fig. 4).

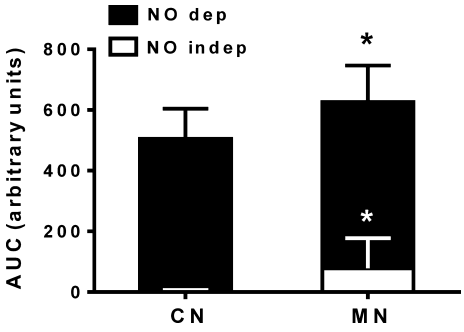

Fig. 4. NO-dependent and NO independent components in pulmonary vasodilatation. Values are means ± S.E.M. for the area under the curve (AUC) for NO-dependent and NO independent relaxation induced by MetCh. Groups are control (CN) and melatonin-treated (MN) lambs. Significant differences ($P \leq 0.05$): *versus CN.

eNOS expression and activity

eNOS expression, at transcript or protein levels, was similar in both groups (Fig. 5 A,B). However, the activating phosphorylation on Serine 1177 was significantly elevated in MN relative to CN (Fig. 5C). Consistently, the stable metabolites of nitric oxide, nitrite/nitrate (NO_x) were significantly increased in MN relative to CN (Fig. 5D).

Pulmonary oxidative stress and antioxidant capacity

MN group showed a significant decrease in the pulmonary oxidative stress markers NT (85 KDa signal), 4-HNE, and 8-isoprostanes, compared to CN (Fig. 6 B,C,D). However, The NT at 55-KDa band showed a tendency to decrease in the MN group ($P = 0.09$, Fig. 6A).

The antioxidant enzymes were analyzed in terms of the transcript, protein and activity levels (Fig. 7). Melatonin treatment increased SOD2 transcript and protein expression in MN relative to CN (Fig. 7A). However, similar levels were observed in SOD1 transcripts (data not shown) and total SOD activity (Fig. 7A) in the lung of both groups. In addition, CAT transcript was increased in MN relative to CN with similar levels of protein expression. Nevertheless, melatonin treatment markedly increased pulmonary CAT activity (Fig. 7B). Further, GPx1 transcript levels were similar, but protein expression was elevated in MN relative to CN. However, total GPx enzymatic activity was similar between groups (Fig. 7C).

Plasmatic oxidative stress, antioxidant capacity, and melatonin levels

At plasmatic level, 8-isoprostanes were significantly reduced in the MN group at day 8 of treatment (Fig. 8A). Further, the FRAP index exhibited a significant increase at any stage of the treatment period in the MN group (Fig. 8B). These were correlated with a significant ~five-fold increase in the plasmatic concentration of melatonin in the MN group relative to controls (Fig. 8C).

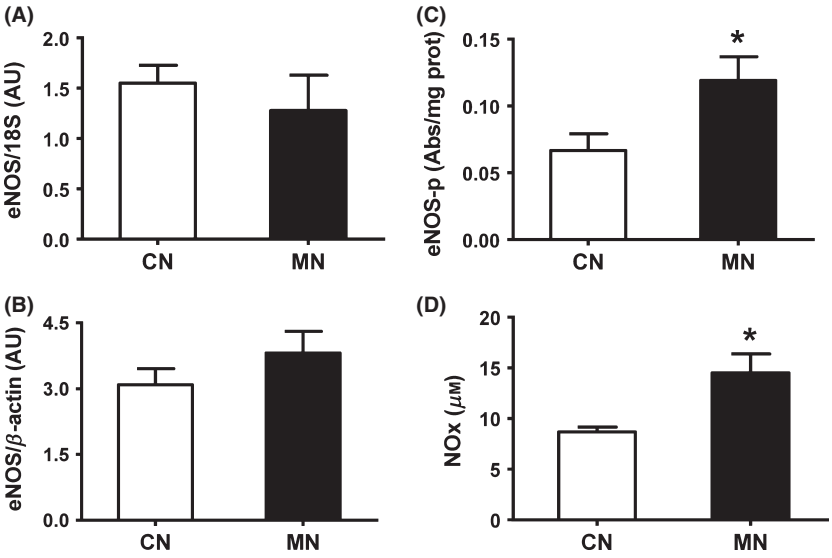

Fig. 5. Components of the pulmonary NO metabolism. Values are means ± S.E.M. for the area under the mRNA (A) and protein (B) and the active phosphorylation (C) of eNOS; and NOx concentrations (D). Groups are control (CN, open bars) and melatonin-treated (MN, closed bars) lambs. Significant differences ($P \leq 0.05$): *versus CN.

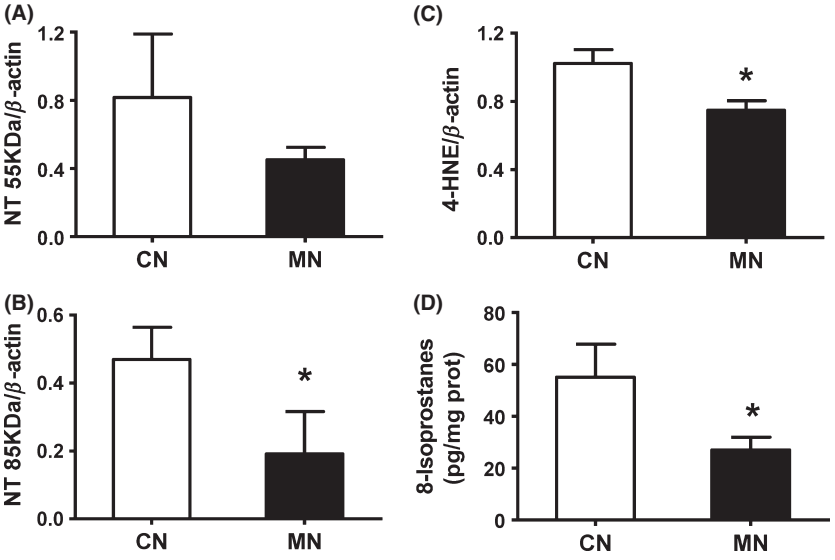

Fig. 6. Oxidative stress markers in the lung. Values are means ± S.E.M. for the levels of NT 55KDa (A) and 85KDa (B), 4-HNE (C) and 8-isoprostanes (D) in neonatal lungs. Groups are control (CN, open bars) and melatonin-treated (MN, closed bars) lambs. Significant differences ($P \leq 0.05$): *versus CN.

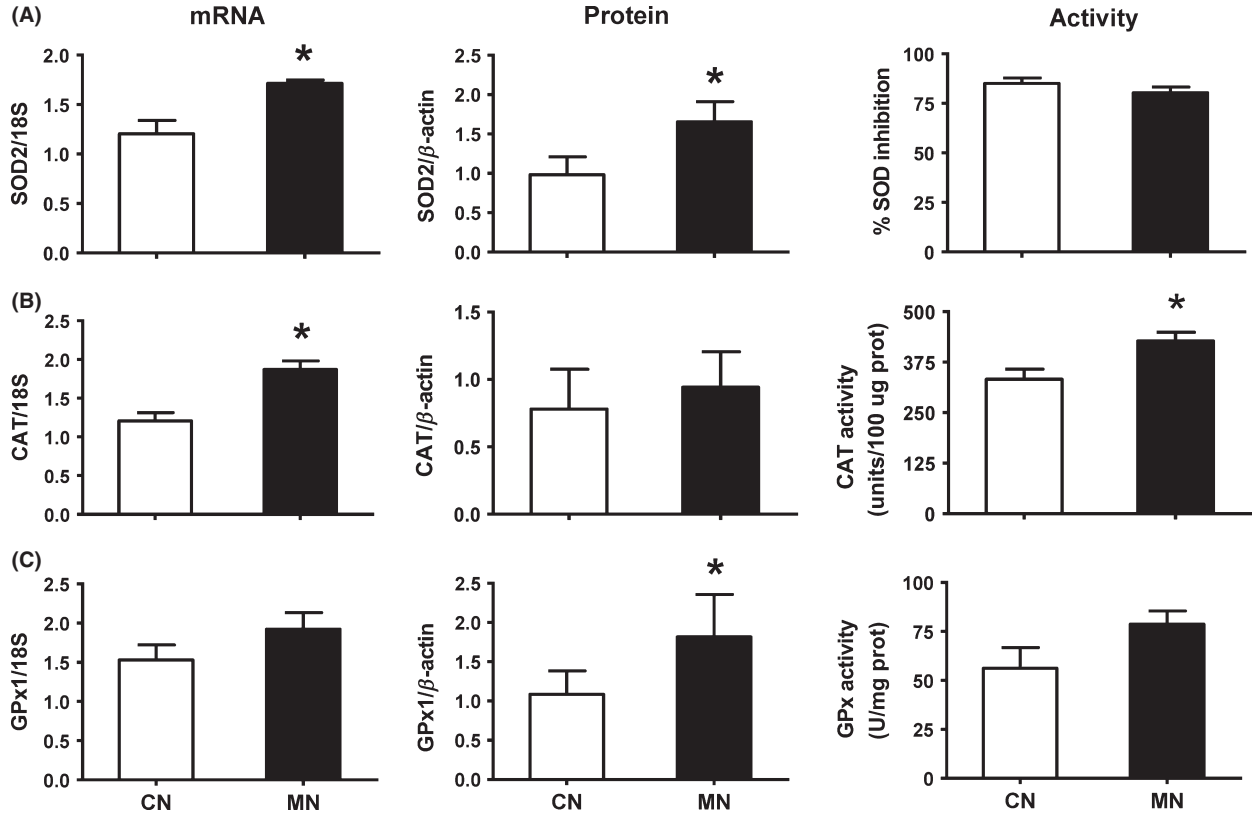

Fig. 7. Enzymatic antioxidant function in the lung. Values are means ± S.E.M. for the levels of mRNA, protein and activity of SOD (A), CAT (B) and GPx (C) in neonatal lungs. Groups are control (CN, open bars) and melatonin-treated (MN, closed bars) lambs. Significant differences ($P \leq 0.05$): *versus CN.

Histomorphometry of small pulmonary arteries

The histological analyses showed that small pulmonary arteries were similar in external diameter in both groups (Fig. 9C). However, melatonin increased arterial luminal area relative to controls (Fig. 9D). Further, the wall thickness was similar in small arteries of both groups (Fig. 9E). Consequently, the wall to lumen ratio, an index of vascular remodeling, was reduced in the MN group (Fig. 9F).

Discussion

The results of this study showed that during the first weeks of life, there are discrete changes in the pulmonary circulation

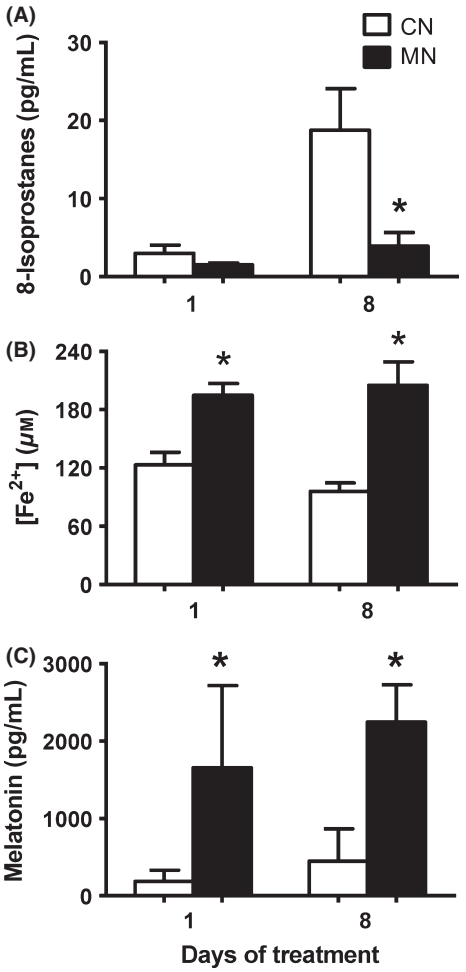

Fig. 8. Plasmatic oxidative stress, reducing capacity and melatonin. Values are means ± S.E.M. for plasma 8-isoprostanes (A) and Fe^{+2} levels (FRAP, B) and melatonin (C) at days 1 and 8 of treatment. Groups are control (CN, open bars) and melatonin-treated (MN, closed bars) lambs. Significant differences ($P \leq 0.05$): *versus CN at equivalent days.

of newborn lambs gestated at high altitude. Moreover, our findings revealed that a postnatal treatment with melatonin decreases pulmonary arterial pressure and vascular resistance at the beginning of the treatment. Further, we showed clear evidence of an improved vascular function related to an increased nitrergic tone, enhanced antioxidant capacity, and lower oxidative stress in the postnatal lung with melatonin treatment. Finally, these outcomes were related with an anti-remodeling effect in the small pulmonary arteries. Therefore, this study supports the concept that melatonin has important antioxidant and beneficial vascular effects in the lung during the postnatal period in chronically hypoxic neonates.

Newborn lambs gestated at high altitude have a greater PAP in the early postnatal period in contrast with lambs gestated at sea level [16]. This is the first study showing daily cardiopulmonary hemodynamic changes in the first 2 wk of life at high altitude. In fact, we showed that at 10–11 days old, there is a slight but significant decrease in PAP. In comparison with normoxic lowland neonates, this is a delayed pulmonary transition, which stabilizes near puberty but in higher PAP levels [3, 52]. Postnatal melatonin treatment seems to ease the transition, inducing an early decrease in pulmonary vascular resistance and pressure. This effect may be due to its vasodilator and/or antioxidant actions. Previous studies have shown that melatonin vasodilates different vascular beds, presumably by MT2 receptor [32, 36, 37, 53]. Further, NO and $\cdot O_2^-$ reaction ($k \sim 7 \times 10^9$ L/mol/s) produces peroxynitrite [54], diminishing NO bioavailability and thus its vasodilator capacity. Therefore, if melatonin scavenge $\cdot O_2^-$, there will be more NO available for vasodilator functions [55], which may be one of the explanations of the decreased PAP at early stages and the enhanced NO-dependent dilatation associated with increased NO products (NO_x) by the end of the treatment [56]. Further, peroxynitrite inhibits eNOS, increases 8-isoprostanes, and promotes vasoconstriction in neonatal lung [57], consistent with our results. In fact, our studies showed similar eNOS expression between groups but an increased eNOS activity.

The early in vivo effects induced by melatonin are transient, but the improved vascular function persists by the end of the treatment. Therefore, we hypothesized that the short-lasting clinical effect may be due to a rapid vasodilatation effect via MT2 receptors, followed by a downregulation of these receptors induced by melatonin [58]. However, the mechanisms commanding these differential in vivo effects should be the focus of future studies to test the role of melatonin receptors.

The endothelium-dependent relaxant agonist (MetCh) showed low responsiveness, evidencing a marked pulmonary vascular dysfunction in high-altitude lambs. Melatonin treatment significantly increased MetCh sensitivity, suggesting an improvement in the endothelial function. As shown in the SNP response curve, the endothelium independent relaxation was also enhanced in the melatonin-treated group, with a higher maximal response and a better vasodilator capacity. These results imply both, an endothelial and muscular-improved vasodilator function, consistent with our findings where we showed enhanced NO-dependent and independent relaxation mechanisms. Moreover, increases in the activating eNOS phosphorylation and in NO metabolites (NO_x) support the idea of a mayor NO bioavailability in the melatonin group. Another possible effect of melatonin is the modulation of ROS-mitochondrial generation. For instance, it has been described that melatonin increases the efficiency of the electron transport chain thereby limiting electron leakage and free radical generation in the mitochondria [59]. Therefore, we might be in front of a double action of melatonin, decreasing ROS generation and improving ROS clearance. These antioxidant effects of melatonin are clearly supported by the decreased oxidative biomarkers and lipid peroxidation products nitrotirosine, 4-HNE, and 8-isoprostanes. Further, the direct effects of melatonin as a scavenger are manifest in the enhancement of the reducing capacity of the plasma and in the reduction of plasmatic 8-isoprostanes, nicely correlating with the plasmatic concentrations of melatonin. Therefore, melatonin is also having systemic effects that may be translated into a better physiological status during neonatal transition. This

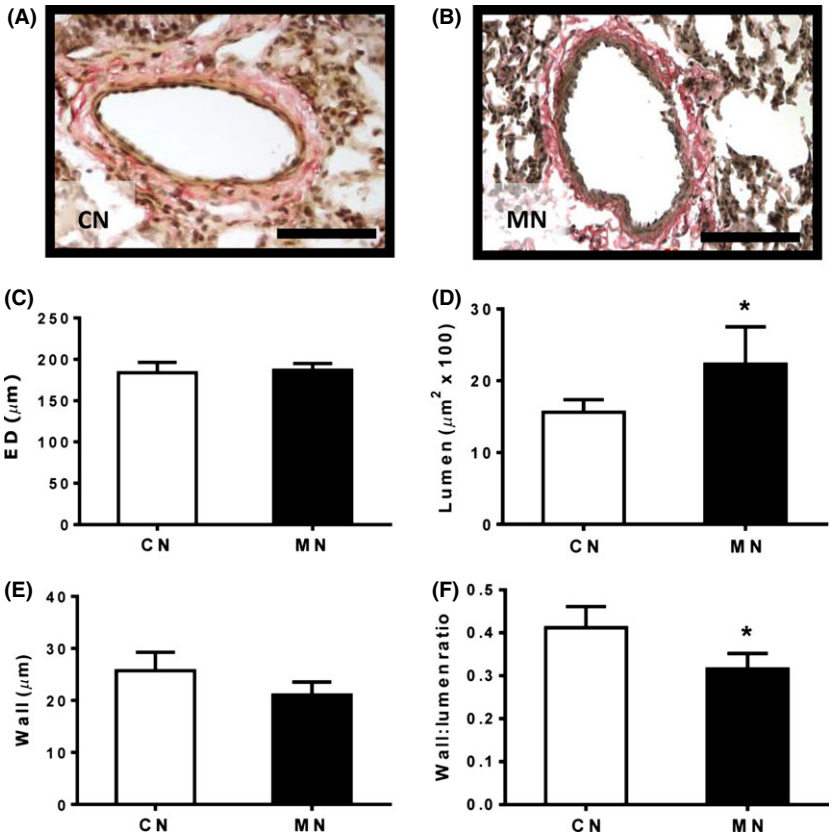

Fig. 9. Morphostructural characteristics of small pulmonary arteries. Representative micrographs (40×) of Hematoxylin–Eosin stained lung sections (A,B), external arterial diameter (ED, C), luminal area (D); wall thickness (E) and wall/lumen areas ratio (F). Groups are vehicle (CN, white bars) and melatonin (MN, black bars) treated. Bar in the micrographs = 100 μm. Values are means ± S.E.M. Significant differences ($P \leq 0.05$): *versus CN.

improvement in the antioxidant capacity without changes in systemic arterial pressure supports melatonin use in a wide range of perinatal disorders [27, 31, 32, 60].

Melatonin can stimulate antioxidant enzymes under normal and oxidative stress conditions [34, 61–67]. In this work, melatonin effectively stimulated CAT, showing a higher activity in pulmonary tissue of the treated group. On the other hand, we found greater levels of the transcript and protein level of SOD2, but not changes in the total activity. These findings may be explained by the fact that melatonin is a negative modulator of SOD1 [34], therefore conserving the SOD total activity. Similarly, the protein induction of GPx1 by melatonin was not associated with increases in total GPx enzymatic activity, which may imply differential effects of melatonin in the GPx isoforms. The antioxidant enzymes' regulation by melatonin is via its G protein-coupled receptors MT1 and MT2 and mediated by second messengers cAMP, phospholipase C and intracellular Ca^{2+} [34, 65] as well as the activation of MAP kinases and transcription factors [68]. Further, melatonin can regulate the calcium/calmoduline pathway through its receptors or via direct binding to calmoduline [69, 70]. Also, melatonin stimulates NFkB an AP-1 transcription factors, which are related to the promoter region of the antioxidant enzymes [71–73]. All these outcomes imply a complex direct regulation of melatonin on the antioxidant enzymes in a receptor dependent and independent manner. Another potential mechanism induced by melatonin is the increase of heme-oxygenase-1 expression, a known vasoactive agent that has vasodilator and antioxidant effects [74]. However, many of the above mechanisms still remain to be confirmed in the pulmonary circulation of neonates.

Although important effects were seen in our study, we did not get clinical effects by the end of treatment. This could be explained by a species-, targeted organ-, or dosing-dependent effects. Indeed, a recent study showed that melatonin attenuates pulmonary hypertension remodeling processes after 4 wk of treatment under hypoxia in adult rats [75]. The latter supports that we may found clinical beneficial effects in pulmonary circulation of neonates, with a higher dose, a longer treatment, and/or an earlier administration of melatonin. In the last couple of years, several reviews have highlighted the effective uses of melatonin against diverse pathological states related to oxidative stress, such as perinatal asphyxia, neonatal sepsis, respiratory distress syndrome, bronchopulmonary dysplasia, retinopathy of prematurity, and intraventricular hemorrhage, among others [31, 60, 76]. In general, these authors concluded that the particular perinatal susceptibility to oxidative stress indicates that the use of melatonin as an antioxidant may improve the neonatal outcome. From this point of view, we are contributing with information about the mechanisms and effects induced by melatonin. Thus, we propose melatonin as a potential adjuvant therapy for PHN, complementing vasodilatatory treatments such as iNO or sildenafil.

In conclusion, even though the newborn gestated and born at high altitude treated with melatonin did not restored clinical parameters of pulmonary hypertension, this work clearly showed positive results in reducing oxidative stress and improving vascular function and

morphology of small pulmonary arteries. It is likely that a multifactorial and complex pathology such as PHN will not respond effectively to a monotherapy [40]. In this line of thinking, we proposed that melatonin is a potential adjuvant drug in a pharmacological combination looking after vasodilatation, preventing excessive proliferation of the tunica media, an restoring oxidative tone. Regarding the incomplete understanding of the pathology, more studies are urgently needed for an efficient treatment of PHN.

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Author contributions

FT, AGC, CM, GE, RVR, AJLL, and EAH conceived and designed the experiments. FT, AGC, CM, GE, MC, MSF, RVR, AJLL, and EAH collected, analyzed, and interpreted the experimental data. FT, AGC, and EAH drafted the article, and all authors revised it critically and approved the final version.

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