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Melatonin long-lasting beneficial effects on pulmonary vascular reactivity and redox balance in chronic hypoxic ovine neonates

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Abstract

Pulmonary arterial hypertension of the neonate (PAHN) is a pathophysiological condition characterized by maladaptive pulmonary vascular remodeling and abnormal contractile reactivity. This is a multifactorial syndrome with chronic hypoxia and oxidative stress as main etiological drivers, and with limited effectiveness in therapeutic approaches. Melatonin is a neurohormone with antioxidant and vasodilator properties at the pulmonary level. Therefore, this study aims to test whether a postnatal treatment with melatonin during the neonatal period improves in a long-lasting manner the clinical condition of PAHN. Ten newborn lambs gestated and born at 3600 m were used in this study, five received vehicle and five received melatonin in daily doses of 1 mg kg^{-1} for the first 3 weeks of life. After 1 week of treatment completion, lung tissue and small pulmonary arteries (SPA) were collected for wire myography, molecular biology, and morphostructural analyses. Melatonin decreased pulmonary arterial pressure the first 4 days of treatment. At 1 month old, melatonin decreased the contractile response to the vasoconstrictors K^+, TX_2, and ET-1. Further, melatonin increased the endothelium-dependent and muscle-dependent vasodilation of SPA. Finally, the treatment decreased pulmonary oxidative stress by inducing antioxidant enzymes and diminishing pro-oxidant sources. In conclusion, melatonin improved vascular reactivity and oxidative stress at the pulmonary level in PAHN lambs gestated and born in chronic hypoxia.

KEYWORDS

chronic hypoxia, melatonin treatment, oxidative stress, pulmonary hypertension of the neonate, reactive oxygen species

1 | INTRODUCTION

Pulmonary arterial hypertension of the neonate (PAHN) is defined as an increase in the mean pulmonary arterial pressure (PAP) above 25 mm Hg at rest. PAHN is usually associated with a degree of hypoxemia, pulmonary hypoxia, and oxidative stress.[1] This condition is characterized by abnormal vascular reactivity and vascular smooth muscle remodeling, yielding to high pulmonary vascular resistance (PVR).[2,3]

PAHN has a prevalence of up to 7/1000 live births in lowlands with high morbidity and mortality.[4,5] However, it is estimated that this syndrome can reach up to 10% of the newborns at highlands (>2500 m).[6,7] Chronic hypoxia during pregnancy and after birth promotes an increased pulmonary

vascular resistance with enhanced cardiac afterload in the neonates. These aspects lead to the thickening of the right ventricle and the pulmonary artery, favoring an increased pulmonary pressure.[7-9]

One of the mechanisms proposed in the etiology of this disease is the pulmonary redox imbalance suffered by these newborns, implying an increased generation of reactive oxygen species (ROS) and/or an impaired antioxidant capacity.[10] At a vascular level, ROS are mainly generated in the mitochondria (complex I and III),[11] the NADPH oxidase and the complex xanthine oxido-reductase.[12] Conversely, a decreased antioxidant capacity may depend on a decreased antioxidant machinery expression and/or activity.

Therefore, antioxidant therapeutic strategies have been proposed to treat PAHN in the first days of neonatal life.[13] Thus, melatonin has been highlighted by the experimental evidence, due to its effects as a direct scavenger and inducing antioxidant enzymatic capacity.[1,14-16] In addition, it has been shown that melatonin has vasodilator properties[1] and may modulate pro-oxidant sources[17] in the neonatal lung. These facts are relevant because the limited treatments accepted for PAHN are palliative and expensive: oxygen supplementation associated with inhaled oxide nitric (iNO) and the extracorporeal membrane oxygenation (ECMO).[18] Nevertheless, iNO fails in many cases, and ECMO has important adverse effects.[19] Furthermore, several experimental therapeutic approaches are being tested, such as prostacyclin analogues, endothelin-1 receptor antagonists, phosphodiesterase 5 inhibitors, and stimulators of guanylate cyclase, but their use has not yet been approved for PAHN.[18,20] For this reason, it is necessary to search for new alternatives that decrease PAP, helping to improve the oxidative tone and preserving the pulmonary vasoreactivity, without generating adverse effects attributed to the treatment. In this study, we hypothesized that melatonin decreases pulmonary pressure, improves vascular function, and enhances antioxidant capacity, while decreasing pro-oxidant sources in lung of PAHN lambs.

Therefore, this study aims to test melatonin as a possible alternative therapy for PAHN, based on a translational model of newborn lambs. We used lambs gestated and studied in chronic hypobaric hypoxia, with daily oral doses of melatonin during the first three postnatal weeks.

2 | MATERIALS AND METHODS

All animal care and experimentation procedures were approved by the Committee of bioethics of the Faculty of Medicine, University of Chile (CBA # 0761 FMUCH), and carried out in accordance with international standards of the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

2.1 | Surgical preparation and in vivo experiments

All lambs were instrumented at 3 days old under general anesthesia (ketamine 10 mg kg^{-1}–xylazine 0.04 mg kg^{-1}, IM) and aseptic procedures. From day 3 onwards, daily monitoring of hemodynamic variables was performed every morning until 29 days old, as previously described.[1,17,21] The pulmonary artery pressure (PAP) was recorded every morning (between 9:00-11:00 hours for 30 minutes) with an acquisition system (PowerLab/8SP System and Chart v4.1.2 Software System, ADInstruments) connected to a computer. In addition, the cardiac output (CO) was determined in triplicate by the thermodilution method with a Swan-Ganz catheter connected to a cardiac output computer (COM-2 model. Baxter). Pulmonary vascular resistance (PVR) was calculated as described previously.[21] All in vivo measurements were performed in conscious, unanesthetized animals, while kept in a comfortable sling.

2.2 | Pharmacological treatments

Ten newborn sheep (*Ovis aries*) were gestated, born and studied in highlands (Putre, 3600 m), and randomly divided into two experimental groups. The control group received vehicle (C, n = 5, 1.4% ethanol 0.5 mL kg^{-1}) and the treated group received melatonin (M, n = 5, melatonin 1 mL kg^{-1} in 1.4% ethanol 0.5 mL kg^{-1}), in daily doses from day postnatal 4-21. In order to maintain the normal rhythmicity of the melatonin secretion, all doses were given at 20:00. Animals were monitored from day 3 till day 28 of age, to evaluate the cardiopulmonary function before, during, and after the treatment.

2.3 | Pharmacokinetics of melatonin

Blood samples were collected through a central venous catheter during the study. The blood sampling times were as follows: 0, 15, 30, 45, 60, 90, 120, and 180 minutes after oral administration, and 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes after an intravenous bolus. The intravenous administration was performed once, to evaluate the pharmacokinetics of melatonin at the end of the treatment (21 days old). The blood fractionation of samples was carried out at 5000 × *g* for 5 minutes, and plasma samples were stored at −80°C. The quantitative determination of plasma melatonin concentrations was performed by enzyme-linked immunosorbent assay (Melatonin ELISA KIT, ENZO cat # ENZ - KIT150 - 0001), according to the manufacturer's guidelines. All plasma samples were analyzed in duplicate and reported as average.

For the pharmacokinetic variables, we determined the time point (t_{max}) to reach maximum plasma concentrations

(C_{max}). Furthermore, the halftime was determined by the method of residual[22] and the area under curve (AUC) was calculated using the trapezoidal method.[23] The distribution volume (Vd) and clearance (CL) were estimated from individual linear regression, following the equations Vd = dose/C_0, and CL = ke × Vd. Finally, the bioavailability was calculated as ($AUC_{0-\infty\ oral}/AUC0_{-\infty\ IV}$) × 100. C_0 is considered as the basal plasma concentration at time 0. Pharmacokinetic parameters were calculated by Microsoft Excel add-in, PKSolver.[24]

2.4 | Ex vivo pulmonary arterial function

At 29 days old, the lambs were euthanized with an overdose of sodium thiopental (100 mg kg^{-1}, slow infusion I.V). At postmortem, lungs were weighed and rapidly dissected to obtain samples for wire myography, molecular biology, and histology studies.

The dissection and mounting of the SPA were carried out as described previously.[1] Briefly, 150-300 μm of internal diameter SPA were dissected, cut in 2 mm rings, and mounted in a multichamber wire myograph (DMT 620, Danish Myotechnologies). Vasoreactivity was evaluated by performing concentration-response curves (CRCs) to different vasoactive agents. To assess vasoconstriction responses, CRCs to potassium (K^+), endothelin (ET-1), and thromboxane (U46619) were performed. In addition, vasodilator responses were evaluated in preconstricted arteries (serotonin, 5Ht 10^{-6} mol/L) by methacholine (MetCh), to assess endothelium-dependent vasodilation,and sodium nitroprusside (SNP), to evaluate the muscle-dependent vasodilation. Contractile responses were expressed in terms of wall tension (mN/mm) or percentage of potassium maximal contraction (%K_{max}). Relaxation responses were expressed as a percentage of the contraction induced by 5Ht (%R_{max}).[1]

2.5 | Molecular biology

Total RNA purification from lung tissue, cDNA synthesis, and amplification by PCR was carried out as described previously.[25] Primers were designed for partial amplification of DNA sequences of Eta receptor (forward 5′-GTGGGAATGGTGGGGAAT-3′, reverse 5′-AGAGGAAAACGCCAAAGTCA-3′), ETb receptor (forward 5′-TCATCGGAAACTCCACGC-3′, reverse 5′-ACACATCTAACCCCAAAGG-3′), and 18s rRNA (forward 5′-TCAAGAACGAAAGTCGGAGG-3′, reverse 5′-GGACATCTAAGGGCATCACA-3′) as housekeeping gene. All PCR products were quantified by densitometry (Scion Image Beta 4.02 Win, Scion).

Protein expression of thromboxane A synthase (TXAs), thromboxane receptor (TPα and TPβ), prostaglandin I synthase (PGIs), prostaglandin receptor (Ip), and β-actin was determined in lysates of total pulmonary tissue by Western blot with specific antibodies anti-TXAs (Cayman Chemical, 160715); anti-TP (Cayman Chemical, 101882); anti-PGIs (Cayman Chemical, 160640); anti-IP (Santa Cruz Biotechnology, Sc-365268); and anti-β-actin (AC-15, Thermo Fisher Scientific, MA1-91399). The signals obtained in each Western blot were scanned and quantified by densitometry through a detection device by chemiluminescence (Odyssey Imaging System LI-COR Biosciences).[17]

2.6 | Histomorphometry of small pulmonary arteries

The left lung was perfused, cut in 1 cm^3 pieces, and fixed for 24 hours in paraformaldehyde 4%. Afterward, the tissue was embedded in paraffin, cut in 5 μm slices, and processed with Van Gieson elastic staining for vascular morphometry analysis.[1] Images were captured at 100× and 400× with a digital camera attached to a microscope (Olympus BX-41, Olympus Corporation), and small pulmonary arterial (50-200 μmol/L diameter) dimensions were measured using an image analysis software (Image-Pro Plus 6.2, Media cybernetics, Inc). The diameter and wall thickness were calculated as described elsewhere.[26,27]

2.7 | Xanthine oxidase (XO) activity

Quantification of XO activity in lung tissue was performed using a commercial XO assay kit (XO assay kit, ab102522, Abcam). According to the manufacturer's instructions, the lung tissue was homogenized mechanically with four volumes of assay buffer (assay buffer®); subsequently, the sample was centrifuged at 16 000 × *g* for 10 minutes and the supernatant was isolated. Next, the probe (OxiRED™) was added to the supernatant and incubated at 37°C for 1 hour. Finally, the product of the reaction associated with a specific probe was quantified at an absorbance of 570 nm.[17]

2.8 | NADPH oxidase activity

NADPH oxidase activity was determined based on the rate of consumption of NADPH monitored at 340 nm at 37°C. This test was performed in a solution containing 50 mmol/L phosphate buffer, pH 7.0, 1 mmol/L EDTA, 150 mmol/L sucrose, and homogenate tissue (2-10 μg of protein). The enzymatic reaction was initiated by adding 0.1 mmol/L NADPH. NADPH activity was expressed in μmoles of oxidized NADPH/mg protein/min. Only a slight oxidation of NADPH was detected in the presence of 100 μmol/L apocynin (data not shown), an inhibitor of NADPH oxidase (2.35 oxidized NADPH μmoles/mg protein/min).[28]

2.9 | Mitochondrial superoxide anion generation

Lung tissue was homogenized in five volumes of buffer containing sucrose 70 mmol/L, mannitol 210 mmol/L, HEPES 5 mmol/L, EGTA 1 mmol/L, and BSA 0.5% (p/v), pH 7.2, at 4°C. The homogenized tissue was centrifuged at 1000 × *g* for 10 minutes at 4°C, and the supernatant was collected and centrifuged at 12 000 × *g* for 10 minutes. Then, the sediment was washed and centrifuged at 10 000 × *g* for 10 minutes, and then, it was resuspended in a buffer containing sucrose 70 mmol/L, mannitol 210 mmol/L, and HEPES 5 mmol/L, pH 7.2, at 4°C. This resuspension was immediately used to assess mitochondrial superoxide anion ($O_2^{\cdot-}$) production by measuring the oxidation of DHE 10 μmol/L (470Ex/590Em) for 10 minutes at 37°C.[17]

2.10 | Antioxidant enzyme activity

Antioxidant enzyme activity in pulmonary tissue was measured using superoxide dismutase activity assay kit (KOD; K335-100, Biovision), the test kit of OxiSelect catalase activity (STA-341, Cell Biolabs, Inc) and the kit for glutathione peroxidase assay (703102, Cayman Chemical Company), according to the manufacturer's guidelines. Total protein concentration of each sample was used for standardization purposes as previously described.[1,17]

2.11 | Oxidative stress markers

The oxidative stress markers, 4-hydroxynonenal (4-HNE) and nitrotyrosine (NT), were measured by Western blot. Total proteins (30 μg) were resolved by electrophoresis in SDS-polyacrylamide gels (12%), with running buffer containing TRIS 0.23M, glycine 1.92M, SDS 1% w/v, pH 8.3 and transferred to nitrocellulose membranes under standard conditions. The blots were blocked with 4% nonfat milk in phosphate-buffered saline (PBS) and then incubated with the specific primary antibodies (anti-4-HNE, Abcam Laboratories ab46545, anti-NT, Millipore 05-233). Thereafter, the blots were incubated with specific secondary antibodies and signals were detected by enhanced chemiluminescence (SuperSignal, West Pico Chemiluminescent Substrate; Pierce) and analyzed in a digital Scanner (C-DiGit, LI-COR). In addition, the concentration of 8-isoprostanes in homogenized pulmonary parenchyma was determined with a specific enzyme immunoassay kit (EIA) following the manufacturer's recommendations (Cayman Chemical).[17]

2.12 | Statistical analysis

All data were expressed as mean ± SEM Shapiro-Wilk test was used to evaluate the normality of the data. For in vivo analysis, recordings were expressed as daily averages. For ex vivo analysis, the vasoactive response to potassium was analyzed by a Boltzmann sigmoidal function. The maximal effective tension (E_{max}) and half of the maximal effective concentration (EC_{50}) were determined. The rest of the CRC were analyzed using an equation of best fit agonist response, where vasomotor maximal response was expressed as the percentage of submaximal contractions induced by 5Ht (10^{-6} mol/L). The sensitivity was expressed as pD2 ($-\log EC_{50}$).[21] The cardiovascular data were analyzed by two-way ANOVA and the post hoc test of Newman-Keuls. All other results were compared statistically by an unpaired *t* test. Significant differences were accepted when $P \leq .05$ (Prism 5.0, GraphPad Software).[17]

3 | RESULTS

3.1 | Pharmacokinetic profile of melatonin administration

The basal concentrations of melatonin did not differ significantly in between groups, during the study (85.13 ± 18.20 pg mL^{-1} for C, vs 92.65 ± 12.50 pg mL^{-1} for M). The pharmacokinetic profile and associated variables for melatonin oral administration are shown in Figure 1. Oral melatonin proved to have a kinetic absorption and elimination of first order. The analysis showed a T_{max} of 57.0 ± 6.0 minutes, C_{max} of 9374 ± 558 pg mL^{-1}, halftime of 70.1 ± 5.2 minutes, oral AUC_0 802 498 ± 91 546 pg mL^{-1} min^{-1}, and an absolute bioavailability of 42.9 ± 12.3% Figure 1A. Further, the pharmacokinetic profile of intravenous melatonin administration demonstrated a first-order elimination kinetics Figure 1B.

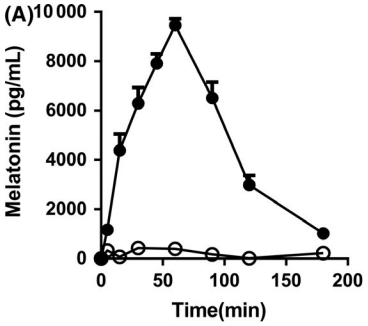
3.2 | Cardiopulmonary variables

At 3 days old, before treatment administration, both experimental groups showed similar values of PAP, PVR, and CO Figure 2. From the second to the fifth day of treatment (5-8 postnatal days), the M group showed a significant decrease in PAP relative to the control group. However, the pressures tended to equalize between the groups toward the end of the treatment (21 postnatal day) Figure 2A. The last week (without treatment) revealed similar mPAP values between experimental groups. Regarding PVR and CO, there were no significant changes between groups along the experimental period Figure 2B,C.

3.3 | Vascular reactivity of pulmonary resistance arteries

3.3.1 | Vasoconstrictor function

The melatonin-treated group (M) decreased the maximum response (E_{max}) to potassium compared with the control



Pharmacokinetic variables: oral (1 mg*Kg^{-1})	
t_{max} (min)	57.0 ± 6.0
Cmax (pg mL^{-1})	9374 ± 558
Halftime (min)	70.1 ± 5.2
AUC $_{0\text{-}180}$ (pg mL^{-1}min^{-1}	802 498 ± 91 546
bioavailability (%)	42.9 ± 12.3

Pharmacokinetic variables: Intravenous (1 mg*Kg^{-1})	
Cmax (pg*mL^{-1})	48 610 ± 11 063
Halftime (min)	18.9 ± 2.2
Vd (L*Kg^{-1})	0.72 ± 0.08
Clearance (L *min^{-1}Kg)	0.0320 ± 0.0063
AUC $_{0\text{-}120}$ (pg *mL^{-1}min^{-1})	1 877 989 ± 418 643

FIGURE 1 Pharmacokinetic profile of melatonin. Pharmacokinetic variables for an oral administration (1 mg Kg^{-1}) of melatonin (A), and pharmacokinetic variables for an intravenous administration (1 mg Kg^{-1}) of melatonin (B). Graphs show values of melatonin concentrations in plasma. Inserted tables show time to reach the maximum concentration (t_{max}), maximal concentration in plasma (C_{max}), halftime, area under the curve (AUC), bioavailability (%), distribution volume (Vd), and clearance. Data are the mean ± SEM Groups are control (C, white circles) and melatonin-treated (M, black circles) lambs

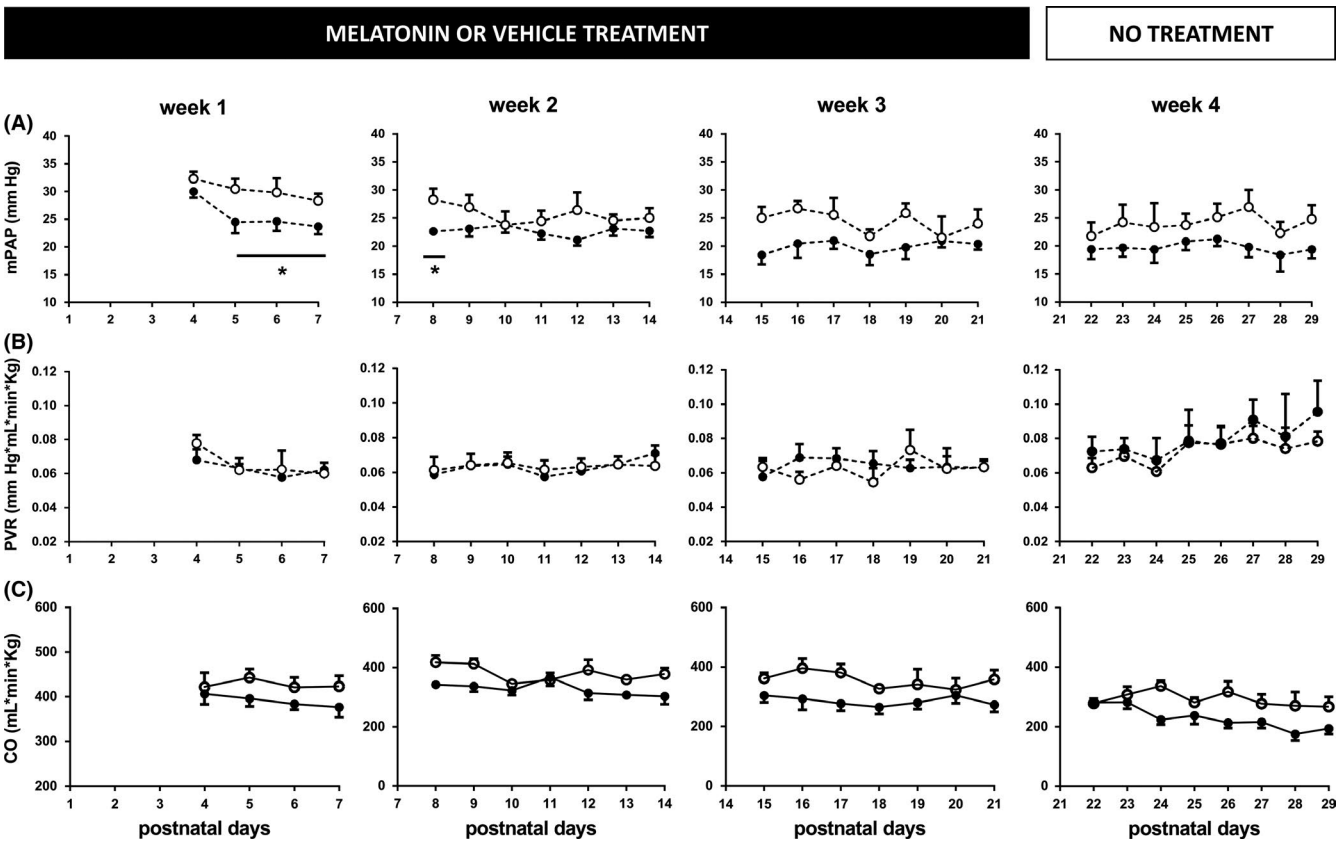


FIGURE 2 Cardiopulmonary Variables. Pulmonary arterial pressure (A, PAP), pulmonary vascular resistance (B, PVR), and cardiac output (C, CO) in newborn sheep gestated at high altitude (3600 m) during the treatment with vehicle (C, white circles) or with melatonin (M, black circle). Values are means ± SEM Significant differences ($P \leq .05$): *vs C at equivalent days

group (C), but the potency was not modified Figure 3A. As well, the constriction response to endothelin-1 showed a decreased maximal response in the melatonin group, while the potency (pD2) was similar to controls Figure 3B. Similarly, the maximal response to the thromboxane mimetic (U46619) was decreased in M relative to C group, while the potency (pD2) was similar in both groups Figure 3C.

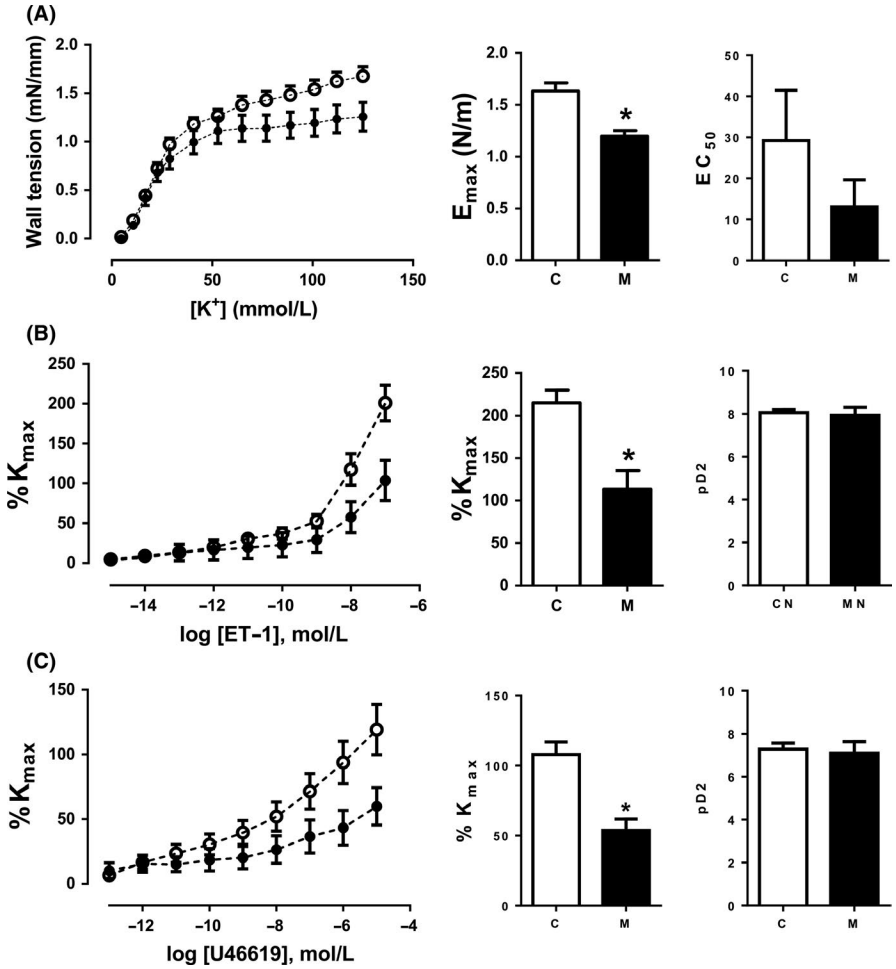


FIGURE 3 Vasoconstrictor function of pulmonary arteries. Vascular response to potassium (A, K^+), to endothelin-1 (B, ET-1), and to thromboxane (C, U46619). Maximal responses (E_{max} or K_{max}) and sensitivity (EC_{50} or pD2) were calculated (inserted histograms). Groups are control (C, open white circles/bars) and melatonin-treated (M, black circles/bars) lambs. Values are means ± SEM Significant differences ($P \leq .05$): *vs C

3.3.2 | Vasodilator function

The endothelium-dependent vasodilator response mediated by methacholine (MetCh) exhibited an enhanced maximal relaxation (% R_{max}) and sensitivity (pD2), in the melatonin-treated group Figure 4A. Furthermore, the smooth muscle-dependent vasodilation induced by sodium nitroprusside (SNP) showed an enhanced maximal relaxation in the melatonin-treated group. However, the sensitivity to SNP was similar in both groups Figure 4B.

3.4 | Vasoactive pathway expression

Transcripts of endothelin receptor A (ETa) showed no significant difference between groups, while the expression of endothelin receptor B (ETb) significantly increased in the melatonin-treated group Figure 5A. In addition, protein expression of the thromboxane synthase (TXs) and the TX receptor subclass β (TPβ) was similar between analyzed groups. However, the receptor subclass α (TPα) decreased its expression with melatonin in comparison with the control group Figure 5A.

Proteins related to vasodilator pathways, such as eNOS and PGI2s, showed similar pulmonary levels between groups. However, sGC and IP receptor increased their protein expression with melatonin treatment Figure 5B.

3.5 | Histomorphometry of pulmonary resistance arteries

Histomorphometric analysis of SPA showed an increased internal and external diameter in the melatonin-treated group Figure 6A,B. However, the media and adventitia thicknesses were similar between groups Figure 6C,D. In addition, the luminal/vascular areas ratio and the media cellular density were similar in C and M Figure 6E,F.

3.6 | Redox balance and oxidative stress

Melatonin decreased the generation of $O_2^{\cdot-}$ from xanthine oxidase (XO), NADPH oxidase, and mitochondria in comparison with the control group Figure 7A. In addition, melatonin induced the antioxidant activity of superoxide dismutase (SOD) and catalase, while the glutathione peroxidase activity remained conserved relative to the control group Figure 7B. Finally, melatonin treatment decreased 8-isoprostanes and NT levels Figure 7C. In

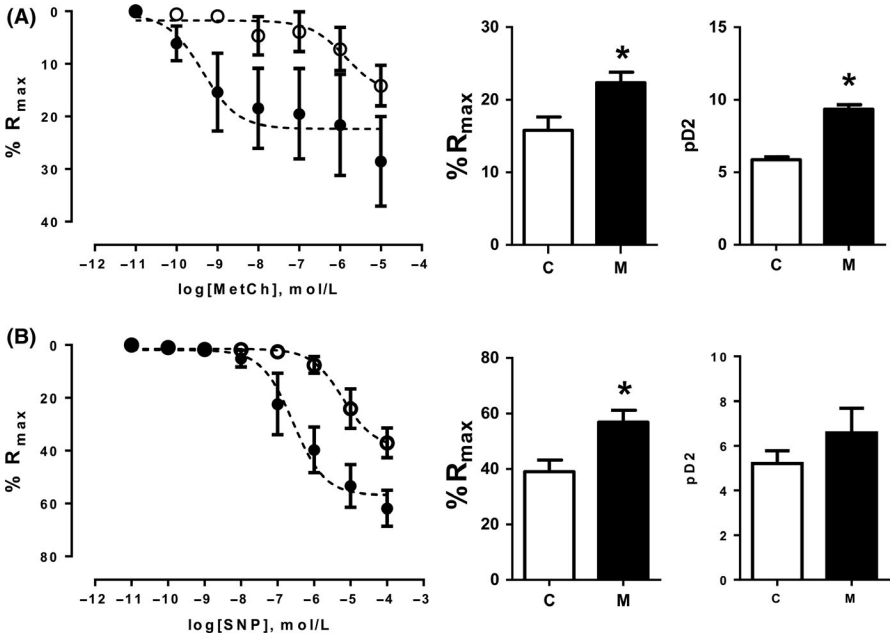


FIGURE 4 Vasodilator function of pulmonary arteries. Vascular response to methacholine (A, MetCh) and to sodium nitroprusside (B, SNP). Maximal responses ($\%R_{max}$) and sensitivity (pD2) were calculated (inserted histograms). Groups are control (C, white circles/bars) and melatonin-treated (M, black circles/bars) lambs. Values are means ± SEM Significant differences ($P \leq .05$): *vs C

contrast, MDA levels were similar between the analyzed groups Figure 7C.

4 | DISCUSSION

The results of this study showed that a daily administration of melatonin at a dose of 1 mg kg^{-1} for the first 3 weeks of life reduced the pulmonary arterial pressure in the 1 week of treatment, increased the pulmonary arterial vasodilator mechanisms ex vivo, and improved the pulmonary redox balance. These results show a long-lasting reversal of the endothelial dysfunction characteristic of PAHN, improving vasodilatory pathways dependent of vascular endothelium and smooth muscle. In addition, melatonin decreased pulmonary $O_2^{\cdot-}$ generation and enhanced the antioxidant enzymatic capacity, therefore improving the pulmonary oxidative balance.

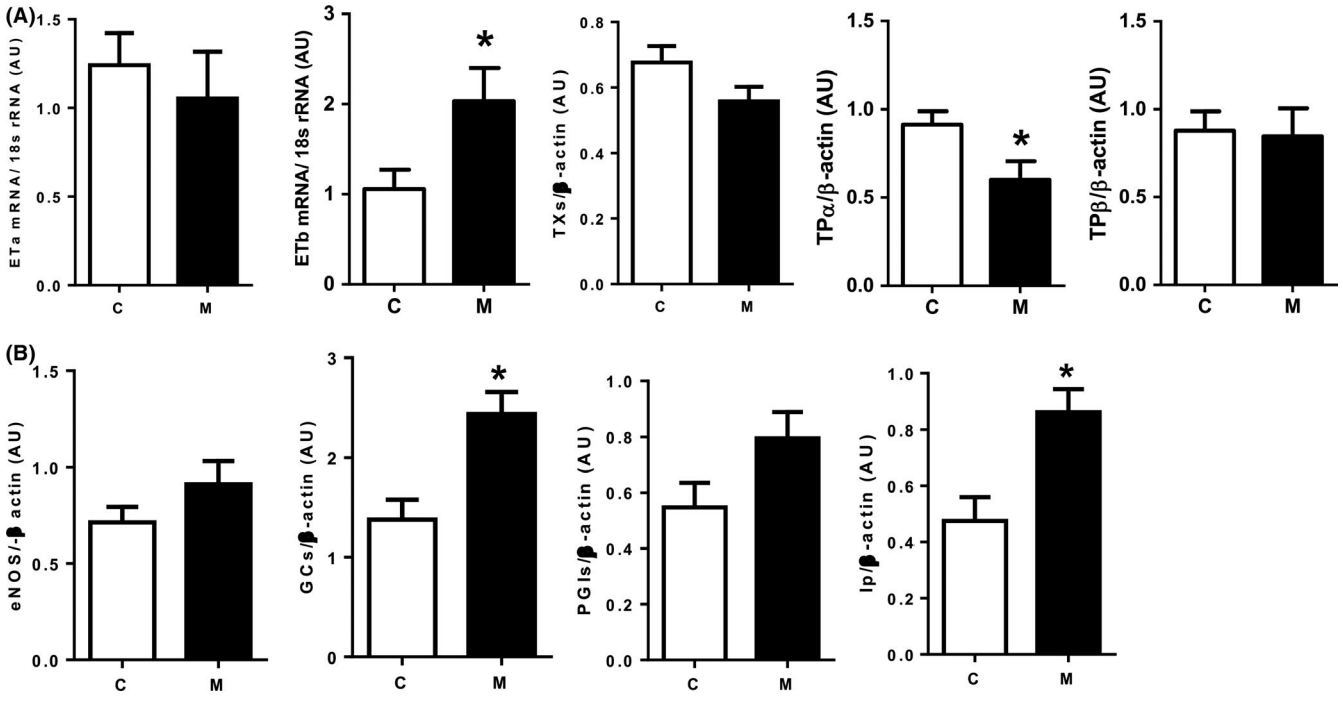


FIGURE 5 Vasoactive pathway mRNA and protein expression. Expression of mRNA and proteins involves in vasoconstrictor (A) and vasodilator (B) pathways. Groups are control (C, white bars) and melatonin-treated (M, black bars) lambs. Values are means ± SEM Significant differences ($P \leq .05$): *vs C

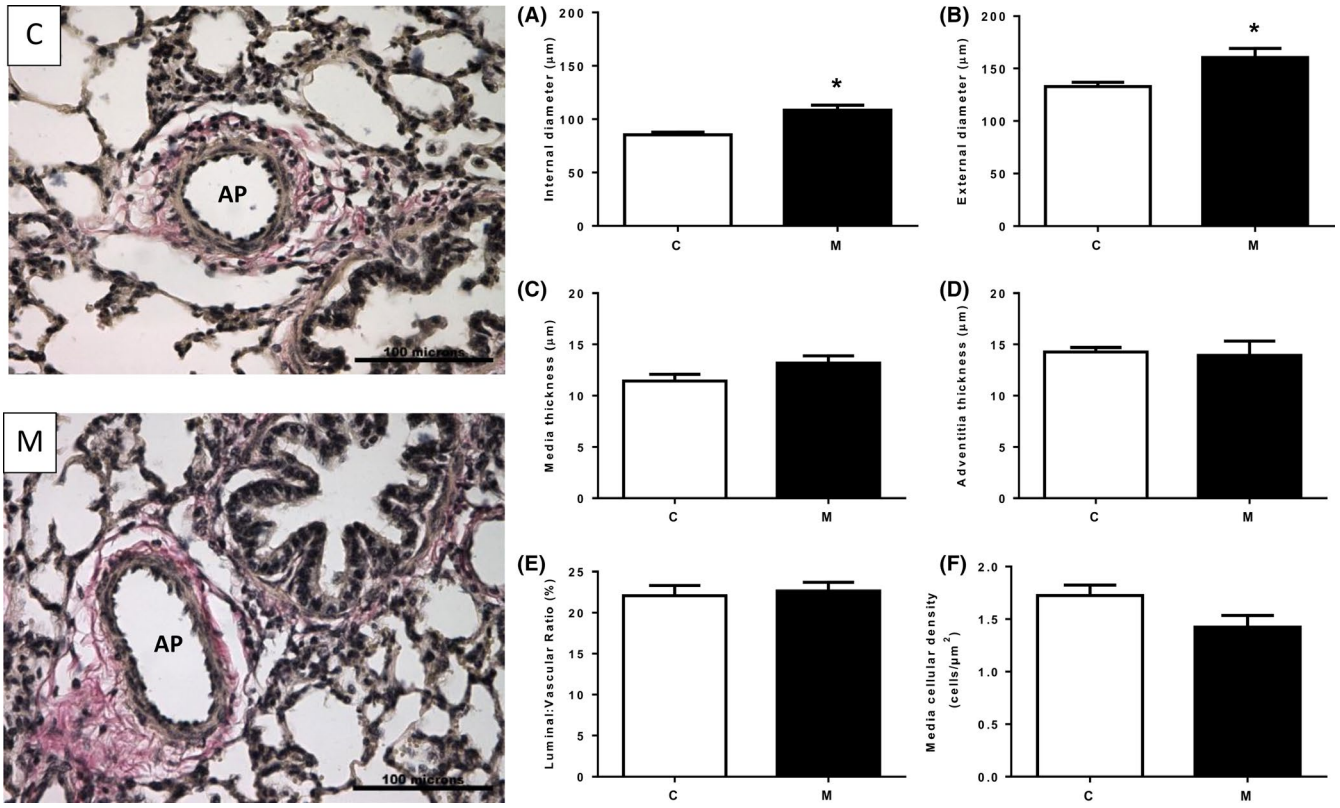


FIGURE 6 Morphostructural characteristics of pulmonary arteries. Internal diameter (A), external diameter (B), media thickness (C), adventitia thickness (D), luminal area/vascular area ratio % (E), and media cellular density (F). Representative micrographs (40×) of lung sections with Van Gieson elastic staining are shown. Bar in the micrographs = 100 µm. Groups are control (C, white bars) and melatonin-treated (M, black bars) lambs. Values are means ± SEM Significant differences ($P \leq .05$): *vs C

This study demonstrates, for the first time, the pharmacokinetics of oral melatonin in newborn sheep. Oral melatonin was absorbed and eliminated by first-order kinetics as it has been previously demonstrated in humans, in dose ranges from 0.3 to 100 mg. Furthermore, melatonin showed pharmacokinetic variables similar to the ones describe for humans,[29] which supports our newborn sheep model as a translational model, from a pharmacological perspective.

Our data demonstrate a high absolute bioavailability of melatonin of 42%, higher than previous studies documenting between 10% and 33% of bioavailability.[30] The high bioavailability obtained in our study can be an effect of the species, caused by differences in absorption, distribution, metabolism, or excretion of the drug. However, the exact causes remain to be established.

The *in vivo* cardiovascular recordings show a pressure above 25 mm Hg in both experimental groups, confirming that our experimental model generates PAHN.[1,21,26] This is the first work that studied hemodynamic variables in control and melatonin-treated lambs during the first postnatal month in a neonatal model at high altitude. Melatonin administration achieved an acute effect, reducing PAP during the first 5 days of treatment. Also it managed to keep the PAP close to 20 mm Hg even when the treatment was removed. This result is relevant because the neonates gestated and born in highlands have a delayed pulmonary transition relative to newborns gestated and born in lowland.[9] Although PAP is similar at the end of the experimental period between groups, the initial days of low PAP may be determinant in improving the pulmonary vascular function seen in our findings once the treatment is finished.

The possible mechanisms by which melatonin induces the acute fall of PAP during the first 4 days of treatment must certainly involve a reversal of the vasoconstrictor tone induced by hypoxia. Our results support this fact since the group treated with melatonin decreased the contractile capacity of the pulmonary arteries. This indicates that the contractile function, determined by either the amount of muscle or by voltage-dependent vasocontractile mechanisms, is being modulated by melatonin. Also, melatonin decreases the maximum contractile response to endothelin-1 by increasing differential expression of the ETb receptor without changes in the expression of ETa receptor. This does not clarify the mechanisms involved in melatonin actions, but previous studies highlight ET1-ETa-ETb pathway as one of the main vasocontractile determinants in hypoxia-induced pulmonary arterial hypertension.[31] Furthermore, ETb receptor is preferentially located at the endothelium, where it contributes with at least two effects:

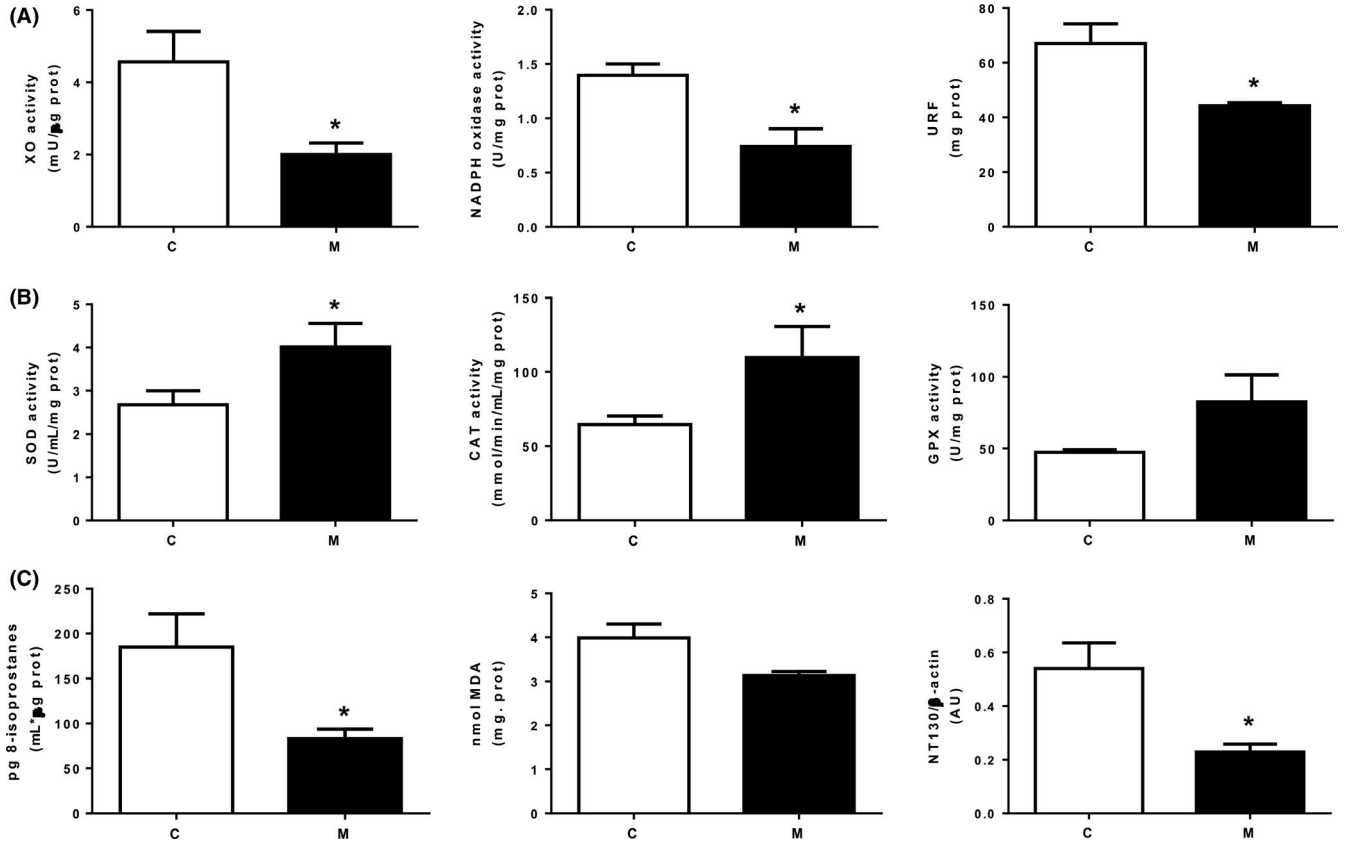


FIGURE 7 Redox balance in lung. Pro-oxidant sources (A): Xanthine oxidase, NADPH oxidase, and mitochondrial activity as an estimation of $O_2^{\cdot-}$ generation. Antioxidant enzymatic activity (B): activity of SOD, CAT, and Gpx. Oxidative stress markers (C): 8-isoprostanes, MDA, and NT130. Groups are control (C, white bars) and melatonin-treated (M, black bars) lambs. Values are means ± SEM Significant differences ($P \leq .05$): *vs C

increase the clearance of ET-1 and increase the dependent endothelium vasodilator mechanisms.[32,33] In addition, melatonin decreases maximum vasocontractile response to thromboxane in resistance arteries, decreasing the Tpα receptor expression. Although both Tp receptors (α and β) have similar affinities for the TXA_2,[34] this Tpα reduction may occur by a decrease of the COX_2-TXs-TXA_2 pathway. Interestingly, the vasocontractile prostanoid pathway is increased under hypoxic condition,[35] therefore, melatonin aids to diminish pulmonary vasoconstrictor tone in PAHN.

The endothelium-dependent and endothelium-independent vasodilator functions increased with melatonin. One likely explanation for this functional improvement is that melatonin is diminishing oxidative tone in the pulmonary circulation, improving the eNOS function and increasing NO bioavailability.[1,12] Further, a decreased oxidative tone improves the functionality of soluble guanylate cyclase soluble (sGC) by activating cGMP-dependent vasodilation.[36] Both alternatives involve lessening the oxidation of BH4 and sGC.[12] The mechanisms by which melatonin has an antioxidant effect are diverse. Firstly, it is a potent ROS scavenger, secondly, it induces antioxidant enzymes expression[1] and activity as evidenced in this study,and thirdly, it induces the activation of NFkB and AP-1[37] via receptors MT1-MT2.[38] Further, in this study, we showed that melatonin also decreases the generation of $O_2^{\cdot-}$ from mitochondria, NADPH oxidase, and xanthine oxidase. Interestingly, previous studies have shown that melatonin has a mitochondrial protective effect, enhancing its bioenergetics and oxidative phosphorylation efficiency.[39] In addition, melatonin inhibits the activation of the NADPH oxidase, and decreases the expression and membrane distribution of the regulatory subunits p47-phox and p67-phox.[40] Although the exact mechanism by which melatonin is decreasing $O_2^{\cdot-}$ generation is unknown, our data are consistent that there is a decrease in the three most important vascular sources as shown previously.[17] These results determine a marked decrease in pulmonary oxidative stress, as demonstrated by the markers 8-isoprostanes and NT.[41] Finally, our findings showed that melatonin increased the size of the small pulmonary arteries, which may be an effect of increased arterial distensibility accompanied by increased vasodilation.[32]

Considering the unique susceptibility to oxidative stress of newborns, and the association with neonatal morbidity, the proposal of antioxidant treatments has been taken seriously in the last years.[42-44]

In conclusion, this work clearly showed the antioxidant and vasodilator effects of melatonin. Although we did not detect any clinical improvement after treatment removal, our results suggest that melatonin could be used as a coadjuvant treatment for PAHN to improve pulmonary vascular function and decrease oxidative stress. Therefore, combining melatonin with current accepted treatments, such as iNO, may improve the clinical outcome of the neonates.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

AG-C and EAH drafted the article. AG-C, AC, EGF, EF, CG-C, SAA, GE, RVR, AJLL, and EAH conceived and designed the experiments. AG-C, AC, EGF, EF, CG-C, SAA, GE, RVR, AJLL, and EAH collected, analyzed, and interpreted the experimental data.

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