J. Pineal Res. 2014; 57:33–42
Doi:10.1111/jpi.12141

Journal of Pineal Research

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin improves cerebrovascular function and decreases oxidative stress in chronically hypoxic lambs

Abstract: Chronic hypoxia during gestation and delivery results in oxidative stress and cerebrovascular dysfunction in the neonate. We assessed whether melatonin, a potent antioxidant and potential vasodilator, improves the cerebral vascular function in chronically hypoxic neonatal lambs gestated and born in the highlands (3600 m). Six lambs received melatonin (1 mg/kg per day oral) and six received vehicle, once a day for 8 days. During treatment, biometry and hemodynamic variables were recorded. After treatment, lambs were submitted to a graded FiO_2 protocol to assess cardiovascular responses to oxygenation changes. At 12 days old, middle cerebral arteries (MCA) were collected for vascular reactivity, morphostructural, and immunostaining evaluation. Melatonin increased fractional growth at the beginning and improved carotid blood flow at all arterial PO_2 levels by the end of the treatment ($P < 0.05$). Further, melatonin treatment improved vascular responses to potassium, serotonin, methacholine, and melatonin itself ($P < 0.05$). In addition, melatonin enhanced the endothelial response via nitric oxide-independent mechanisms in isolated arteries (162 ± 26 versus 266 ± 34 AUC, $P < 0.05$). Finally, nitrotyrosine staining as an oxidative stress marker decreased in the MCA media layer of melatonin-treated animals (0.01357 ± 0.00089 versus 0.00837 ± 0.00164 pixels/μm^2, $P < 0.05$). All the melatonin-induced changes were associated with no systemic cardiovascular alterations in vivo. In conclusion, oral treatment with melatonin modulates cerebral vascular function, resulting in a better cerebral perfusion and reduced oxidative stress in the neonatal period in chronically hypoxic lambs. Melatonin is a potential therapeutic agent for treating cerebrovascular dysfunction associated with oxidative stress and developmental hypoxia in neonates.

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Key words: cerebral circulation, chronic hypoxia, melatonin, neonate, nitric oxide, oxidative stress

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Received March 7, 2014;
Accepted April 25, 2014.

Introduction

Perinatal hypoxia is a challenging condition in current obstetric and pediatric medicine. This is due to the short- and long-term devastating effects, particularly in the central nervous system, resulting in cell death and neuronal function alteration [1, 2]. Up to 3–4 per 1000 worldwide human births are exposed to intrapartum hypoxia, which is correlated with neurologic morbidity and mortality [3]. Conversely, an important number of newborns are exposed to hypoxic conditions, either by cardiorespiratory problems, environmental hypoxia, or both. Thus, there are more than 140 million permanent high-altitude inhabitants, a population where pregnant women and their babies are exposed to developmental chronic hypoxia [4, 5]. This is a major public health matter as these babies not only present health problems in the neonatal period, but also end up with a higher risk of developing cardiovascular and cerebral dysfunctions later in life [2, 6]. Despite the magnitude of the problem, still there is no definitive and effective therapy to prevent or reverse the effects of perinatal hypoxia.

The vascular response to chronic hypoxia in the neonatal brain is complex and not well understood [7, 8]. Hypoxia initiates an acute vasodilator response in the perinatal period [9–11], which increases with vascular maturation [12]. Interestingly, there is evidence of successful high-altitude long-term hypoxia acclimatization, with increases in carotid blood blow [13] and no compromise in cerebral oxygenation [9]. However, this adaptation is correlated with a significant intra-uterine growth restriction [4, 14] and altered vascular functions [13, 15–17]. Moreover, the responses to high-altitude long-term hypoxia may effectively program cerebrovascular diseases at adulthood [18] as vascular development and function require a finely controlled environment [6]. For instance, high-altitude hypoxia modulates adrenergic- and serotonergic-mediated signal transduction in the cerebral vasculature [15] with decreased endothelium-dependent relaxation [16].

Most of the research on perinatal hypoxia and cerebral circulation is performed under acute hypoxic conditions ranging from minutes to hours, but few studies assess chronic hypoxia during development. One of the mechanisms by which hypoxia induces damage is as a result of

the increased generation of reactive oxygen species (ROS) by an incomplete reduction of oxygen [19–22]. Relatively low concentrations of ROS are necessary to operate as signaling molecules in the normal regulation of cerebral vascular tone. However, the increased ROS generation may overwhelm the antioxidant endogenous capacity and determines oxidative stress in the cerebral circulation, leading to changes in blood vessels function and structure [23, 24], and therefore increased risk of cerebrovascular disease. Thus, ROS may induce endothelial injury and consequently inhibition of vasodilating pathways [25–27]. In this way, oxidative stress-related mechanisms underlie development of cerebral diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), stroke, attention deficit, and hypereactivity disorders, among others [22, 28]. Hence, efforts on developing treatments have focused on appropriate antioxidant treatments with divergent results [29, 30].

Melatonin is a multiple-task-hormone [31] with several potential beneficial effects on cellular and organ functions. One of the most relevant and novel roles of melatonin is its antioxidant capacity; working in association with its metabolites as potent scavengers, or modulating the antioxidant capacity by enhancing the antioxidant enzymes expression and activity [32–34]. Moreover, melatonin is a strong neuroprotective agent in hypoxic and ischemic processes, reducing neuronal death, memory impairment, and cognitive dysfunctions presumably through its anti-inflammatory and antioxidant properties [35–42]. In addition, melatonin may have important roles promoting vasodilatation in cerebral and systemic circulations [43–45]. Furthermore, melatonin has shown a wide range of tolerance and few adverse effects, in either human or animal models [46, 47]. These features have suggested melatonin as a promising neuroprotective agent [29], which has already been tested in neurodegenerative disorders [48], where ROS play a key role. However, the mechanisms by which melatonin exerts its cerebrovascular effects are still not completely understood, particularly in cerebral vessels under hypoxic conditions.

Therefore, in this study, we hypothesized that oral treatment with melatonin is able to decrease oxidative stress, improve endothelial function, and enhance cerebral perfusion in chronic hypoxic neonates.

Materials and methods

The Ethics Committee of the Faculty of Medicine, University of Chile approved all experimental procedures of this study (protocol CBA N°. 0398 FMUCH). All the studies on animals followed the ARRIVE guidelines and were performed according with 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 that had been conceived, gestated, born and studied at Putre Research Station (3600 m), International Center for Andean Studies (INCAS), University of Chile, were used in this study. These neonates were randomly divided into two groups, one with postnatal treatment of oral melatonin (MN, n = 6, 1 mg/kg per day) and another with oral vehicle (CN, n = 6, 0.5 mL EtOH 1.4% per kg per day) during 8 days starting at day 4 of life. The doses were prepared and given daily at 18 hr by the same operator.

Surgical preparation and in vivo experiments

All the lambs were surgically prepared at 3 days of age for in vivo experimentation. Briefly, lambs were anesthetized with a ketamine (10–15 mg/kg im)–xylazine (0.1–0.2 mg/kg im) association and additional local subcutaneous infiltration of 2% lidocaine. Polyvinyl catheters were placed into the descending aorta and inferior vena cava via femoral vessels, and a blood flow transducer (3S Flowprobe; Transonic Systems, Ithaca, NY, USA) was installed on the left carotid artery. The surgical procedure lasted about 30 min and after a brief recovery period, the neonate was returned to a pen with her mother. After surgery, biometric parameters (body weight, fractional growth rate, biparietal diameter [BPD], and crown-rump length) and hemodynamic variables (systemic arterial pressure, cardiac output, heart rate, and carotid blood flow [CBF]) were recorded every morning (Powerlab/8SP System and Chart 7 Software; ADInstruments, New South Wales, Australia; connected to a PC). In addition, systemic and carotid vascular resistances were calculated. Treatment with melatonin or vehicle began at 4 days of age, and after 7 days of treatment, the animals were subjected to a graded oxygenation protocol. To induce changes in FiO_2, a transparent loosely tied polyethylene bag was placed over the animal's head and a controlled mixture of air, N_2 and CO_2 was passed. The aortic PO_2 (PaO_2) was increased in 3 min steps of 10 mmHg until reaching levels near to 140 mmHg, followed by a stepwise decrease in arterial PO_2 to near 30 mmHg [49]. The recorded hemodynamic variables such as systemic arterial blood pressure, cardiac output, and CBF were correlated with aortic PaO_2 changes.

Ex vivo and in vitro experiments

At 8 days of treatment (12 day old), lambs underwent euthanasia with an overdose of sodium thiopentone (100 mg/kg slow i.v.) for the collection of left and right middle cerebral arteries (MCA). Both MCA were dissected and processed for wire myography and histology.

MCA vascular reactivity

Two millimeter rings of MCA were dissected and mounted on a wire myograph (DMT 610 Danish Technologies, Aarhus, Denmark), maintained at 39°C, and aerated with 95% O_2–5% CO_2. Optimal diameter was obtained stretching the vessel in a stepwise manner to a standardized tension equivalent to a physiological transmural pressure of 80 mmHg [50]. Concentration–response curves (CRCs) were analyzed in terms of sensitivity and maximal responses by fitting experimental data to a dose–response sigmoid equation (Prism 5.0; GraphPad Software, La

Jolla, CA, USA). Contractile responses to increasing concentrations of K^+ (4.72–64.86 mM) were expressed in terms of tension (mN/mm), while constriction responses to serotonin (5HT, 10^{-10}–10^{-5} M), a thromboxane mimetic (U46619, 10^{-15}–10^{-7} M) were expressed as a percentage of a submaximal contraction with 20 mM K^+. Relaxation responses to the nitric oxide donor sodium nitroprusside (SNP, 10^{-10}–10^{-3} M), methacholine (MetCh, 10^{-11}–10^{-5} M), and melatonin (Mel, 10^{-10}–10^{-4} M) were expressed as a percentage of serotonin submaximal (5HT 10^{-6} M) preconstriction. Sensitivity was calculated as pD2, where pD2 = −log[EC50], with EC50 being the concentration at which 50% of the maximal response was obtained.

MCA histology and immunohistochemistry

Middle cerebral arteries rings were immersed-fixed with 4% paraformaldehyde for 24 hr at 4°C, followed by washes and conservation in PBS + sodium azide 0.1% at 4°C until paraffin inclusion. Hematoxylin–eosin and van Gieson staining were performed on 10-μm slides. 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 [51, 52]. In addition, immunohistochemical detection of endothelial nitric oxide synthase (eNOS) and nitrotyrosine (NT) was performed with commercial antibodies (eNOS: n°610296, BD Transduction Laboratories (www.bdbiosciences.com); NT: 05-233; EMD Millipore Corporation, Billerica, MA, USA), and labeling was developed by a HRP-diaminobenzidine kit (Envision TM+, Dako, Glostrup, Denmark). Slides were digitally acquired at 40× and analyzed as specific color-pixel count per area. This method is based on a digital selection of a color spectrum relative to a positive control. For mark quantification, intima and media layers were selected and measured using Adobe Photoshop (CS5 extended version 12.0, San Jose, CA, USA). The mark intensity (pixels) was then divided by the area (μm^2) of the MCA layers (pixels/μm^2).

Data and statistical analyses

For the correlation analyses between hemodynamic variables and arterial PO_2, a linear regression fit and Pearson correlation was performed. Further, the slope and *y*-intercept were calculated. For the ex vivo analyses, the vascular response to potassium was analyzed using the Boltzmann sigmoidal analysis, and the maximal effective tension (Emax) and the half maximal effective concentration (EC50) were determined. All other CRCs were analyzed using an agonist-response best-fit equation, where the maximal vasomotor response was expressed as percentage of the submaximal contraction induced by K^+ [20 mM] or 5Ht [10^{-6} M] (%Kmax for constriction and %Rmax for relaxation, respectively). The vascular sensitivity was expressed as pD2 (−logEC50) [49]. Differences in the vascular responses to methacholine were compared by calculating the area under the curve and the NO dependent and independent contribution as published elsewhere [50]. Data are expressed as means ± S.E.M. Ratios and percentages were arcsine-transformed prior to statistical analysis. All data were compared statistically by an unpaired *t*-test unless otherwise stated. Significant differences were accepted when $P < 0.05$ (Prism 5.0; GraphPad Software).

Results

Both control and melatonin-treated neonates had similar biometry and cardiovascular data at day 0. In addition, both groups showed comparable daily weight gain

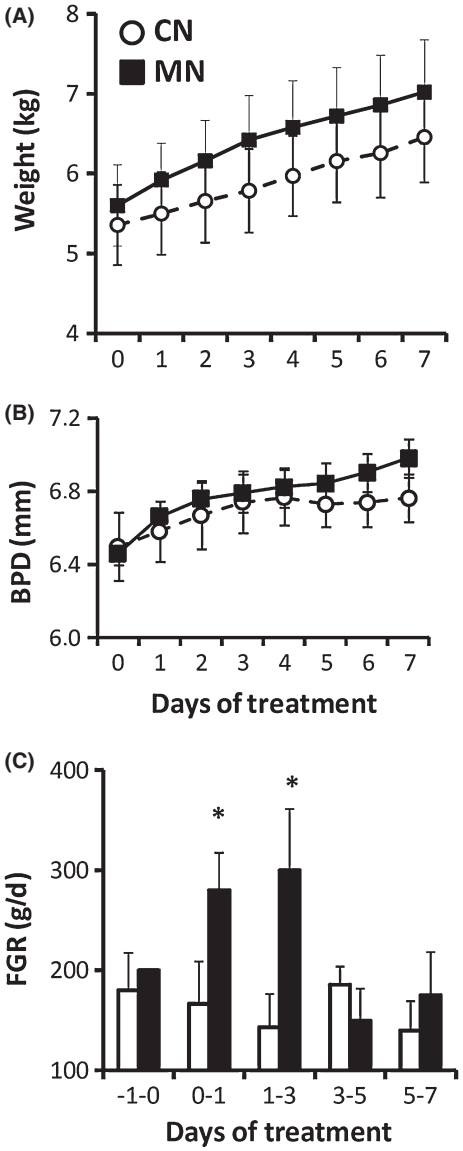

Fig. 1. Neonatal Biometry. Weight gain (A), biparietal diameter (BPD, B), and fractional growth (FGR, C) during the days of treatment. Groups are vehicle (CN, dotted line, open circles, bars) and melatonin (MN, solid line, squares, bars) treated. All measurements were taken during the morning. Values are means ± S.E.M. Significant differences ($P < 0.05$): *Melatonin (MN) versus Vehicle (CN).

(Fig. 1A) and BPD (Fig. 1B) during treatment. However, when expressed as fractional growth, melatonin-treated animals increased significantly during the first 3 days of treatment (Fig. 1C).

The systemic cardiovascular variables and arterial blood gases in the vehicle group were similar to previously reported [49, 51, 53]. In addition, both groups of neonates have similar values in arterial blood gases, systemic arterial blood pressure, cardiac output, and systemic vascular resistance (Table S1 & Figure S1).

During in vivo treatment period, CBF tended to increase in the melatonin-treated animals relative to the control group, reaching a significant difference at day 6 of treatment (Fig. 2A). This effect was also reflected in carotid vascular resistance, where melatonin induced a significant decrease from day 6 of treatment (Fig. 2B).

In addition, after 1 wk of treatment, and during the graded FiO_2 protocol, the CBF was significantly higher at any given PO_2, with correlation functions that were significantly different for the *y*-intercept between groups (Fig. 2C). In addition, melatonin-treated neonates showed a significantly lower CVR at any arterial PO_2 relative to controls, with differences in the slope and the *y*-intercept in between groups (Fig. 2D).

When assessing the vascular SMC function in isolated MCA, melatonin treatment decreased the maximal contractile capacity in response to potassium with similar EC_{50} between groups (Fig. 3A). In contrast, melatonin-treated animals have an enhanced MCA maximal response to serotonin, a potent vasoconstrictor in the cerebral circulation (Fig. 3B). Further, melatonin-treated animals decreased the sensitivity to the thromboxane mimetic U46619, but reached a similar maximal responses relative to controls (Fig. 3C).

In addition, we assessed the vasodilator function in these vessels. Both groups showed similar responses to SNP, a NO donor used to evaluate the muscular dependent NO-vasodilator pathway (Fig. 4A). However, melatonin treatment increased the endothelium-dependent vasodilatation, by markedly enhancing the methacholine sensitivity (Fig. 4B). As well, in vivo melatonin treatment increased the maximum relaxation induced by melatonin in isolated MCA (Fig. 4C). When dissecting the cholinergic endothelium-dependent vasodilator mechanisms with additional blockade with L-NAME, NO-dependent vasodilatation was not affected, but NO-independent vasodilatation was enhanced by postnatal melatonin (Fig. 5A). In addition, immunodetection of eNOS in the MCA slices showed similar levels in both groups (Fig. 5B).

Morphometric analysis of the MCA revealed similar vessel size (Fig. 6A,B) and wall dimensions in vehicle and melatonin-treated animals (Fig. 6A,C). In contrast, immunostainning against nitrotyrosine, as an oxidative stress marker, revealed a diminished mark in melatonin-treated animals relative to controls (Fig. 7).

Discussion

In this study, we showed that a neonatal treatment with melatonin given orally improved cerebral vascular function in chronically hypoxic newborns lambs, increasing the CBF and modifying vascular function of the MCA. Moreover, we demonstrated that the vasodilator function was improved by nitric oxide-independent mechanisms. The latter was related to a decreased oxidative stress in the walls of the MCA. Notwithstanding, morphostructural characteristics of the cerebral arteries were maintained. Importantly, all these changes took place without any systemic hemodynamic modifications, such as hypotension or tachycardia.

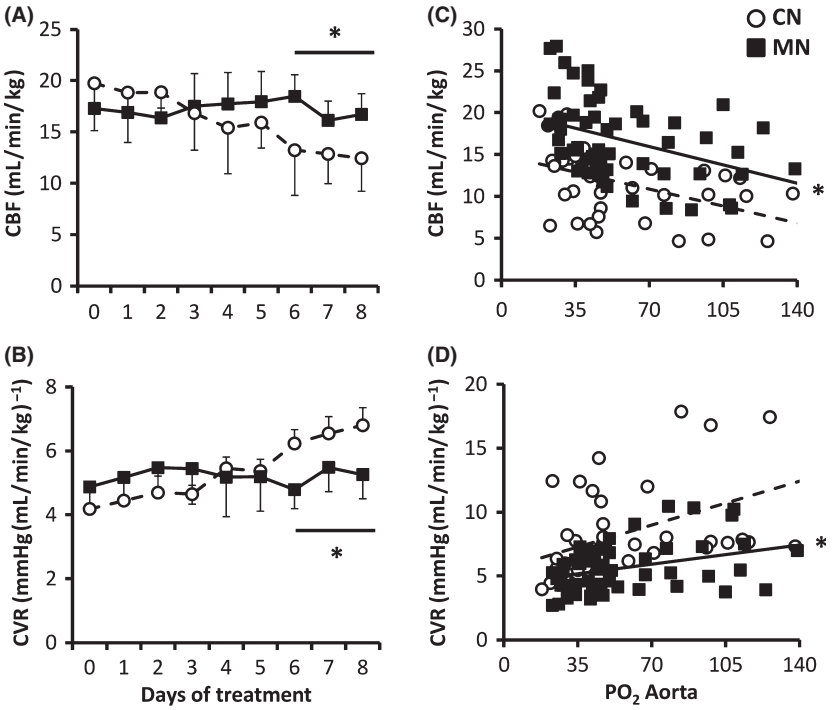

Fig. 2. Neonatal carotid hemodynamics. Carotid blood flow (CBF, A) and resistance (CVR, B) during the days of treatment. Correlation plot and functions for aortic PO_2 and CBF (C); CN: $y = -0.0576x + 14.902$ versus MN: $y = -0.0625x + 20.343$, $P < 0.05$ for *y*-intercept. Correlation plot and functions for aortic PO_2 and carotid vascular resistance (CVR, D); CN: $y = 0.049x + 5.5421$ versus MN: $y = 0.0215x + 4.4115$, $P < 0.05$ for slope and *y*-intercept. Groups are vehicle (CN, dotted line, open circles) and melatonin (MN, solid line, squares) treated. All measurements were taken during the morning. Values are means ± S.E.M. Significant differences ($P < 0.05$): *Melatonin (MN) versus Vehicle (CN).

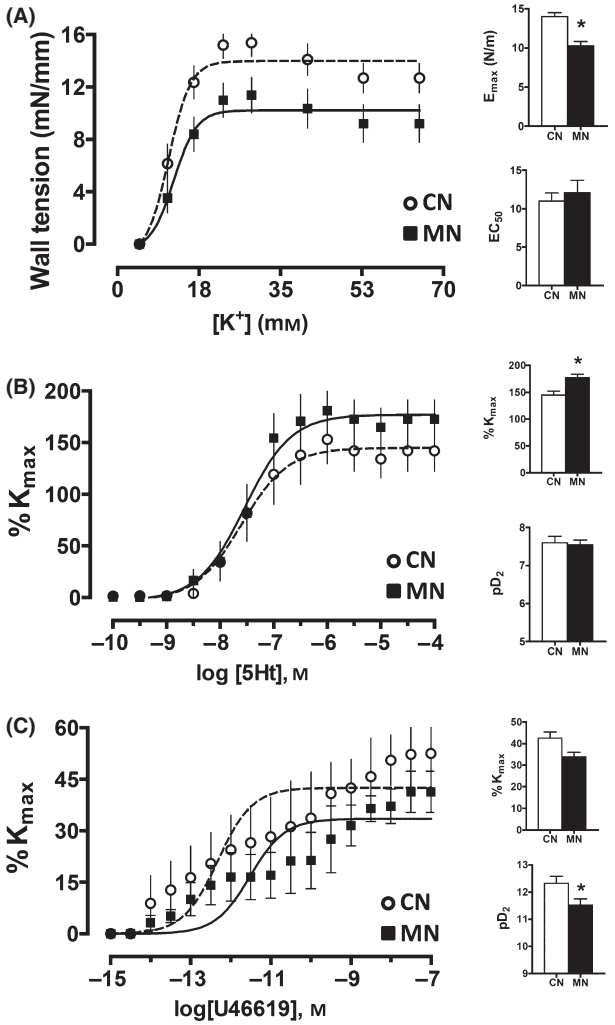

Fig. 3. Middle cerebral arteries (MCA) vasoconstrictor function. Concentration-response curves, maximal response (Emax or % Kmax) and sensitivity (EC50 or pD2) to potassium (K^+, A), serotonin (5Ht, B), and a thromboxane mimetic (U46619, C). Groups are vehicle (CN, dotted line, open circles, bars) and melatonin (MN, solid line, squares, bars, MN) treated. Values are means ± S.E.M. Significant differences ($P < 0.05$): *Melatonin (MN) versus Vehicle (CN).

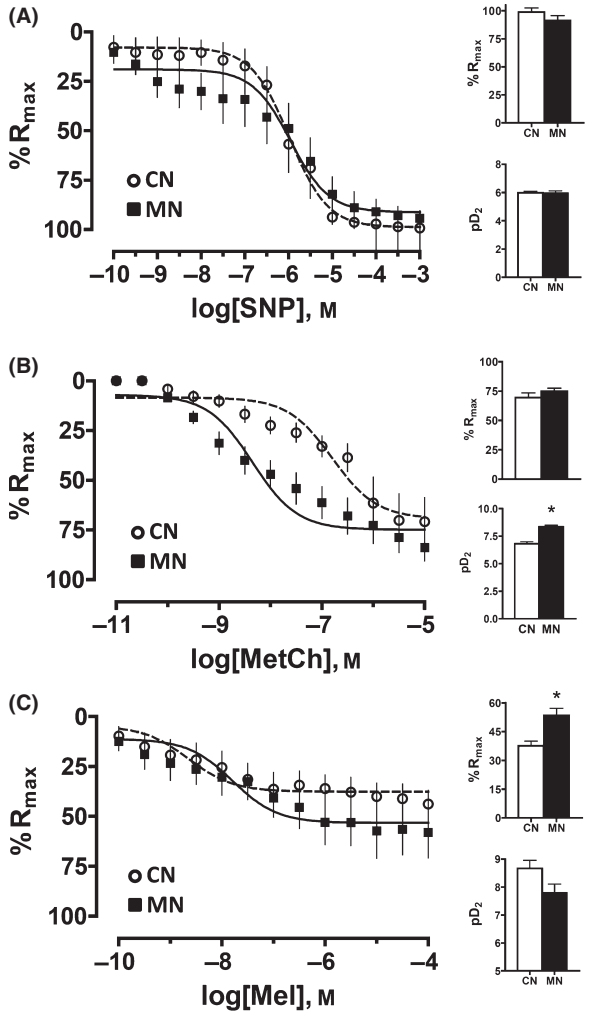

Fig. 4. Middle cerebral arteries (MCA) vasodilator function. Concentration-response curves, maximal response (%Rmax) and sensitivity (pD2) to sodium nitroprusside (SNP, A), methacholine (MetCh, B) and melatonin (Mel, C). Groups are vehicle (CN, dotted line, open circles, bars) and melatonin (MN, solid line, squares, bars) treated. Values are means ± S.E.M. Significant differences ($P < 0.05$): *Melatonin (MN) versus Vehicle (CN).

The antioxidant capacity of melatonin includes its scavenger action and other indirect actions such as induction of the expression and/or activity of antioxidant enzymes and decreasing of pro-oxidant enzymes [32–34, 54]. In addition, studies in knockout mice have shown that the melatonin neuroprotective effects during ischemia appear to be independent of MT1 and MT2 receptors [36]. Further studies have shown that under neonatal hypoxia, melatonin may protect vascular and nervous tissue through its antioxidant, anti-inflammatory, and anti-apoptotic effects [41]. Melatonin exerts its action as a ROS scavenger by a cascade of reactions involving the participation of several of its successive metabolites [33, 55]. It is possible that the ability of melatonin to scavenge ROS can significantly suppress the inhibitory effect that these molecules exert on endogenous NO, enhancing its bioavailability and consequently resulting in a more efficient vasodilator effect [56, 57]. In addition, in vitro experiments have shown that melatonin scavenges NO in the presence of oxygen and it possibly interacts with peroxynitrite rather than NO alone [58]. Melatonin also influences both antioxidant enzyme activity and cellular messenger RNA levels of endogenous antioxidant enzymes [54], such as superoxide dismutase (SOD), both MnSOD and CuZnSOD, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase [54, 55, 59, 60]. The relative importance of each of the direct and indirect antioxidant mechanisms by which melatonin acts in vivo is not fully understood and it might depend on the experimental conditions [61, 62]. Our results indicate that melatonin effectively increased cerebral perfusion after 5 days of treatment. On the basis of the in vivo effects, we can infer that melatonin may have reduced superoxide anion and thus increased NO bioavailability and enhanced the cerebral vasodilatation.

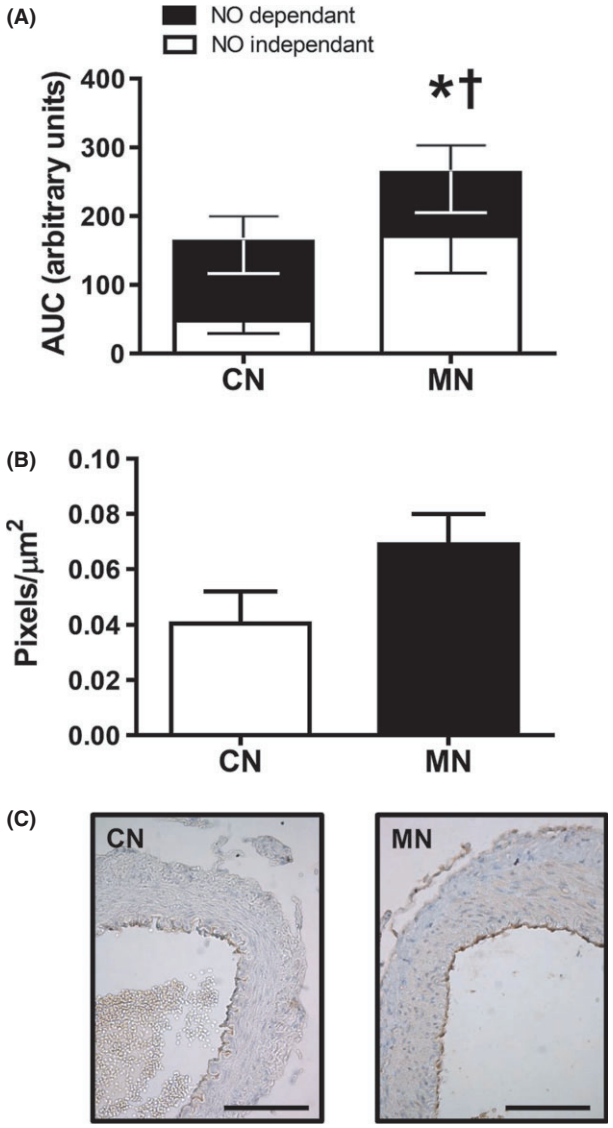

Fig. 5. Partial contribution of NO-dependent and NO-independent mechanisms to the middle cerebral arteries (MCA) relaxation. Area under the curve (AUC) for methacholine (MetCh)-induced relaxation (complete bar with positive S.E.M.), the AUC for MetCh-induced relaxation following treatment with LNAME (NO-independent component, white bar with negative S.E.M.), and the remaining AUC after MetCh with LNAME (NO-dependent component, black bar with negative S.E.M) (A). Endothelial eNOS expression by immunohistochemistry (B) and representative micrographs 40× (C). Groups are vehicle (CN) and melatonin (MN) treated. Bar in the micrographs = 100 μm. Values are means ± S.E.M. Significant differences ($P < 0.05$): *Melatonin (MN) versus Vehicle (CN) for total endothelial-dependent relaxation, †Melatonin (MN) versus Vehicle (CN) for NO-independent relaxation.

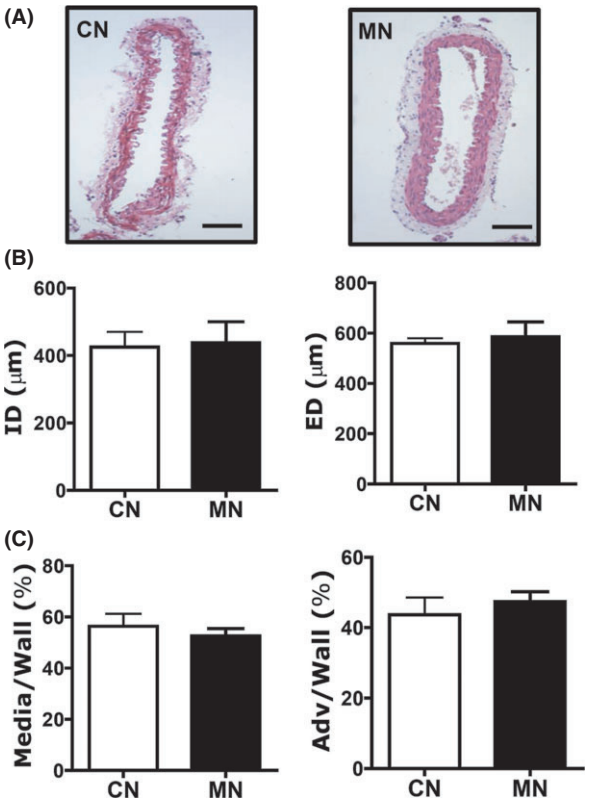

Fig. 6. Morphostructural characteristics of middle cerebral arteries (MCA). Representative micrographs (10×) of hematoxylin–eosin-stained MCA (A); calculated internal arterial diameter (ID, B) and external diameter (ED, B); media/wall percentage area (C) and adventitia/wall percentage area (C). Groups are vehicle (CN, white bars) and melatonin (MN, black bars) treated. Bar in the micrographs = 100 μm. Values are means ± S.E.M.

In the present study, the ex vivo and in vitro findings clearly showed that the improved vasodilator function by melatonin in the cerebral vasculature was due to a rise in nitric oxide-independent mechanisms. In fact, in our ex vivo experiments, melatonin showed a direct vasodilator effect on cerebral arteries. This outcome suggests that melatonin may exert a vasodilator effect, through a specific receptor pathway, the adrenergic blockade, and/or various others NO-independent mechanisms. For instance, it has been shown in rat caudal arteries that MT2R activation results in vasodilatation [63, 64]. Further, Torres-Farfan et al. [43] showed direct, MT1R- and MT2R-independent vasodilator effects of melatonin in fetal cerebral arteries, which might be related with a local sympathetic-inhibitory mechanism, able to diminish norepinephrine-induced contraction. In this sense, at least MT1R is highly expressed in bovine MCA [65]. Although we did not quantify expression or activity of MT receptors, in our model, melatonin might be acting by both MTR-dependent and independent pathways.

Endothelial function may contribute to the regulation of vascular smooth muscle tone by producing active molecules such as NO, endothelin-1, thromboxane, prostacyclin, carbon monoxide, and several growth factors. The suppression of prostaglandin production may be another possible mechanism of the melatonin vasodilator effect [63]. Further, melatonin increases the heme-oxygenase-1 expression [66], having synergistic vasodilator and antioxidant effects. These responses are of importance as they induce active vasodilatation as well as they may contribute to antiremodeling processes, particularly at low levels of NO [67]. Although we did not get changes in the MCA

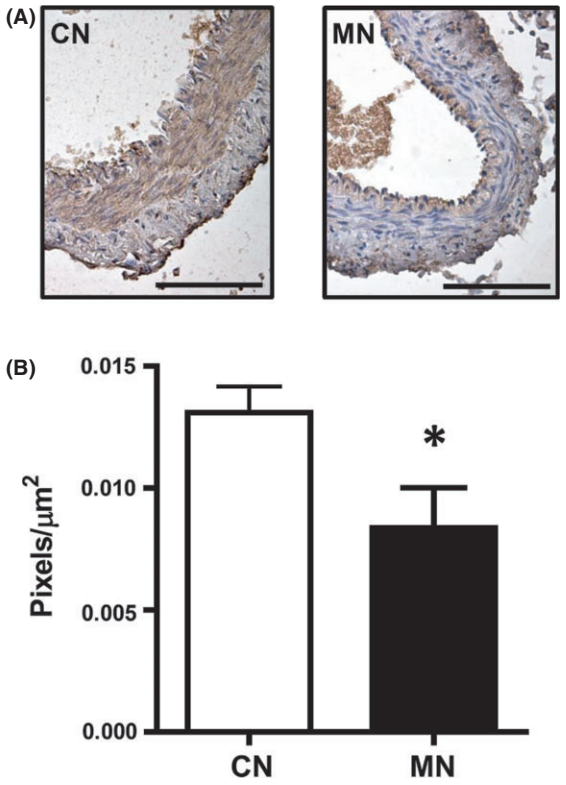

Fig. 7. Oxidative stress in middle cerebral arteries (MCA). Representative micrographs (40×) of nitrotyrosine expression by immunohistochemistry (A) and quantification (B). Groups are vehicle (CN, white bars) and melatonin (MN, black bars) treated. Bar in the micrographs = 100 μm. Values are means ± S.E.M. Significant differences ($P < 0.05$): *Melatonin (MN) versus Vehicle (CN).

media layer thickness with the melatonin treatment, we could argue that the diminished contractile capacity is the response to an established balance toward a vasodilator state, which was evidenced by the decreased responses to potassium and thromboxane.

Nitric oxide synthases activity increased along with gestation, exerting vasodilatatory effects on the brain [68–70]. In fact, endothelial isoform (eNOS) expression increased along pregnancy [69] and is markedly upregulated by hypoxia [70, 71]. In contrast, Wood et al. [72] observed that eNOS and nNOS brainsteam expression decreased in the last third of gestation in fetal sheep. Conversely, Pearce et al. [73] suggested that hypoxic inhibition of NO-induced vasodilatation is attributable largely to attenuation of the specific activity of soluble guanylate cyclase and increased phosphodiesterase activity. The antioxidant effect of melatonin may ease this process and as a result increase NO bioavailability. This is a NO-dependent vasodilatation that would be only evident in in vivo measurements and is independent of eNOS expression and activity, reason why it is not reveal in our ex vivo data. Further, melatonin reduces NO production induced by bradykinin in endothelial cells, by still unknown mechanisms upstream to the interactions of Ca^{2+}-calmodulin with eNOS [74]. In contrast, the in vivo chronic treatment with melatonin improves NO-dependent and NO-independent relaxation but without increasing eNOS expression [75]. This is consistent with our observations, as melatonin increased methacholine sensitivity, mainly by a NO-independent mechanism. The interesting outcomes are that in vivo treatment with melatonin improves cerebral vasodilatation and perfusion, as well as possessing a direct vasodilator effect in MCA.

On the other hand, in intermittent [76] and chronic [16, 73] hypoxic conditions, eNOS expression and function is reduced and a concomitant treatment with melatonin in the former exerts its protective action by restoring the expression of eNOS and NO bioavailability [76]. Chronic hypoxia during development is affecting vascular NO function in the brain, but still remains as an important area to be further explore [77].

We gave 1 mg/kg of melatonin orally once daily and got plasma levels 1–2 order of magnitude higher than controls. Preliminary measurements using radioimmunoassay [78] gave plasmatic levels of 100–300 pg/mL in controls versus 2000–3000 pg/mL in melatonin-treated animals in the near midnight sampling (peak hour). The hemodynamic monitoring was performed every morning (10:00 hr) in our study, at low levels of plasma melatonin. We consider important to avoid potential adverse effects by maintaining the circadian rhythm of melatonin. Melatonin has several functions and probably the most important one is its signaling as biological mediator of seasonal environmental light–dark cycle, fact that needs to be conserved when considering melatonin administration [79].

While the effects of treatment with long-term melatonin have been poorly researched, one study showed that melatonin does not affect the rate of growth and behavior in adulthood [80]. In our study, we noted a small effect in growth at the beginning of the treatment, maybe by an improved oxygen utilization and better redox balance at a cellular level in the hypoxic conditions of our model.

An important benefit of melatonin is the demonstrated low toxicity even at high doses in the perinatal period [47, 81, 82]. In fact, no treatment-related side effects with long-term melatonin therapy in children and adults have been reported [83]. These facts give melatonin advantages over other antioxidant treatments such as vitamin C and E, which have shown adverse effects during pregnancy and the early postnatal period [50, 84–87]. In this study, we were able to administer melatonin in a daily low dose, which implies a safe and easy route of administration at a very low cost.

The exogenous administration of melatonin in experimental models has documented its beneficial effects in reducing cell damage induced by hypoxia, ischemia/reperfusion, and oxidative stress in the central nervous system [35, 37–42]. For instance, melatonin reduces the size of infarcted tissue, decreases apoptosis, and decreases levels of oxidative stress [88, 89]. In newborn mice subjected to cerebral hypoxia, melatonin protects against brain injury, reducing lipid peroxidation products such as MDA and increasing the activity of catalase [90]. In addition, melatonin administration in cerebral hypoxia–ischemia attenuates not only brain damage at short term, but also showed long-term benefits in learning and behavioral disorders [80]. Currently, the most important neuroprotective treatment

in perinatal asphyxia is therapeutic hypothermia and brain cooling. A recent study in piglets showed that the combination of melatonin with cooling may significantly improve neuroprotection in a safe manner [91]. It might be that melatonin is facilitating the perfusion recovery in a decreased oxidant level after cooling. All of the antioxidant-dependent effects are of particular relevance as infants are potentially more prone to generate oxidative stress than other individuals, as they have higher O_2 consumption than adults, lower plasma levels of antioxidants, a decreased enzyme activity, and a potentially immature antioxidant system [83, 92].

In conclusion, this report shows clear evidence in vivo and ex vivo that melatonin is actively vasodilatating cerebral circulation in chronically hypoxic neonates and proposed involved mechanisms. Understanding the pathways by which melatonin performs these beneficial effects is vital for implementing clinical trials and proposing safe and effective treatments in clinical practice. Melatonin appears to be a powerful and effective antioxidant, able to modulate muscular and endothelial function in the MCA and improved cerebral perfusion via NO-independent mechanisms. Although further studies are necessary to have a full understanding of the mechanisms involved, the fact that melatonin has few reported adverse effects, causes important vascular functional improvements and a low cost, makes this molecule a promising and realistic treatment for diseases associated with chronic hypoxia and cerebrovascular dysfunction in the neonatal period.

Author contributions

The experiments in this study were performed in the University of Chile. EAH, RM, CM, RVR, and AJLL conceived and designed the experiments. EAH, RM, CM, GE, MD, SR, JTP, MSF, RVR, and AJLL collected, analyzed, and interpreted the experimental data. All authors drafted the article, revised it critically, and approved the final version.

Acknowledgements

We are grateful for the excellent technical assistance of Mireya Delgado, Carlos Brito and Gabino Llusco.

Funding

This work was supported by National Fund for Scientific and Technological Development (FONDECYT-Chile), Grants number 1110595, 1120605, 1130424, 1140647.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Neonatal systemic hemodynamics.

Table S1. Arterial acid-base status and blood gases.