OBSTETRICS

Potential adverse effects of antenatal melatonin as a treatment for intrauterine growth restriction: findings in pregnant sheep

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BACKGROUND: Intrauterine growth restriction is a condition in which the fetus has a birthweight and/or length <10th percentile for the gestational age. Intrauterine growth restriction can be associated with various causes, among which is low uteroplacental perfusion and chronic hypoxia during gestation. Often, intrauterine growth-restricted fetuses have increased oxidative stress; therefore, agents that decrease oxidative stress and increase utero, placental, and umbilical perfusion have been proposed as a beneficial therapeutic strategy. In this scenario, melatonin acts as an umbilical vasodilator and a potent antioxidant that has not been evaluated in pregnancies under chronic hypoxia that induce fetal growth restriction. However, this neurohormone has been proposed as a pharmacologic therapy for complicated pregnancies.

OBJECTIVES: The aim of this study was to determine the effects of prenatal administration of melatonin during the last trimester of pregnancy on the biometry of the growth-restricted lambs because of developmental hypoxia. Further, we aimed to determine melatonin and cortisol levels and oxidative stress markers in plasma of pregnant ewes during the treatment.

STUDY DESIGN: High-altitude pregnant sheep received either vehicle (n = 5; 5 mL 1.4% ethanol) or melatonin (n = 7; 10 mg/kg^{-1}day^{-1} in 5 mL 1.4% ethanol) daily during the last one-third of gestation. Maternal plasma levels of melatonin, cortisol, antioxidant capacity, and oxidative stress were determined along treatment. At birth, neonates were examined, weighed, and measured (biparietal diameter, abdominal diameter, and crown-rump length).

RESULTS: Antenatal treatment with melatonin markedly decreased neonatal biometry and weight at birth. Additionally, melatonin treatment increased the length of gestation by 7.5% and shifted the time of delivery. Furthermore, the prenatal treatment doubled plasma levels of melatonin and cortisol and significantly improved the antioxidant capacity of the pregnant ewes.

CONCLUSIONS: Our findings indicate that antenatal melatonin induces further intrauterine growth restriction but improves the maternal plasma antioxidant capacity. Additional studies should address the efficiency and safety of antenatal melatonin before clinical attempts on humans.

Key words: gestation, hypoxia, intrauterine growth restriction, melatonin, neonatal biometry, oxidative stress

Intrauterine growth restriction (IUGR) is defined as a condition in which the fetus has an estimated body weight and/or length at <10th percentile for the gestational age.[1] This condition is associated markedly with increased perinatal morbidity and mortality rates.[1] Therefore, IUGR needs careful prenatal and labor monitoring and appropriate neonatal assistance. Furthermore, IUGR programs several organs and systems through epigenetic mechanisms and triggers metabolic and cardiovascular diseases in neonates, juveniles, and adults.[2-5] This is a worldwide relevant condition, because it is estimated that there are 150 million newborn infants per year and that 5-10% of them, depending on the area, will have low birthweight standardized for gestational age.[5,6] Although many efforts have been made in the IUGR-related mechanisms and potential therapies, there is still no preventive or definitive curative treatment for these babies.

IUGR may be attributed to several causes that include oxygen and/or nutrition deprivation and exposure to toxins during gestation.[2] The most common cause is blood flow restriction with low uteroplacental perfusion, which hampers oxygen and nutrient delivery to the fetus. Moreover, IUGR fetuses and their placentas have increased oxidative stress, which is postulated as an important mechanism involved in IUGR and cardiovascular dysfunction.[7-10] Therefore, agents that increase umbilical and/or uteroplacental perfusion and decrease oxidative stress may be an advantageous therapeutic approach. In this context, melatonin is an effective umbilical vasodilator[11,12] and a potent antioxidant[10,13-20] in the perinatal period. These effects of melatonin that are supported by several studies on animal models have led to the proposal of pilot clinical trials to evaluate the effects of maternal melatonin administration on fetal oxidative stress and growth in human pregnancies that are affected by IUGR.[10,21-23] However, the outcomes of studies of antenatal melatonin in humans have not been revealed yet.

Plasma melatonin levels are decreased in complicated pregnancies such as preeclampsia and IUGR.[22,24,25] Moreover, IUGR and premature babies have a delayed development of melatonin rhythmicity.[26] In addition, the maternal alteration of melatonin levels during

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the last one-third of pregnancy also affects the melatonin synthesis in the offspring.[27,28] However, it is unknown, and still in debate, whether the decrease in melatonin is a cause or effect of the pathologic pregnancies.

Gestation under chronic hypobaric hypoxia induces a marked IUGR in human[29,30] and animal models.[31,32] We have developed a sheep model in which neonates experience the development of IUGR and impaired cardiovascular functions that are related to oxidative stress.[20,32-35] Furthermore, a postnatal treatment with melatonin improved pulmonary and cerebral vascular functions that are associated with a significant fall in oxidative stress after 1 week of treatment.[20,35] In the present study, we evaluated whether a treatment with antenatal melatonin during the last one-third of gestation under chronic hypobaric hypoxia would prevent neonatal IUGR in sheep.

Materials and Methods

All animal care, procedures and experimentation were approved by the Bioethics Committee of the Faculty of Medicine, University of Chile (CBA 0398), 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.

Animals and sampling

Twelve time-mated pregnant sheep (*Ovis aries*) at high altitude (Putre Research Station, International Center for Andean Studies, University of Chile, 3600 m above sea level) were bred and maintained under standard housing conditions in the Research Station. Once verified that the fetal development was a single gestation, we randomly allocated melatonin-treated (MM; n = 7; 4 female and 3 male) and vehicle-treated (MC; n = 5; 3 female and 2 male) sheep. MM ewes received melatonin (10 $mg.kg^{-1}$ day^{-1} in 5 mL 1.4% ethanol); MC ewes received 5 mL of 1.4% ethanol, orally at 18 hours in the last one-third of gestation (starting at 100 days; gestation length approximately 150 days). Melatonin administration was at dusk to preserve physiologic rhythmicity with nighttime increases.[36] Maternal plasma samples were taken at 10 and 22 hours at 120, 130, and 140 days of gestation and kept with 0.005% butylated hydroxytoluene at −80°C until use. Pregnant ewes were left to deliver naturally, and no interventions were made during labor. Immediately after delivery, neonates were attended, weighed, and measured (biparietal diameter, abdominal diameter, and crown-rump length).

Plasma melatonin

Plasma levels of melatonin were obtained by radio immunoassay as previously described.[11] Samples were collected under sterile conditions into chilled EDTA tubes (2 mL K+/EDTA; LIP, Ltd, Shipley, West Yorkshire, UK) and analyzed in duplicate at the same time. Plasma samples were extracted with diethyl ether before being assayed. The assay used melatonin antiserum (batch no. 704 8483; Guildhay Antisera Ltd, Guildford, Surrey, UK) and [O-methyl-3H]-labeled melatonin (85 Cimmol/L; Amersham Biosciences AB, Uppsala, Sweden) as a tracer.[11]

Plasma cortisol

Blood samples (4 mL) were collected and placed in chilled polystyrene tubes that contained 200 μL of 0.5 mmol EDTA. The tubes were centrifuged for 20 minutes at 2800*g* at 4°C. Plasma was separated and stored in aliquots at −80°C. Cortisol was measured by specific radioimmunoassay (Cortisol Coat-a-Count; Diagnostic Products Corp, Los Angeles, CA) as previously described.[37]

Plasma 8-Isoprostanes

As a marker of lipid peroxidation, analysis of 8-isoprostanes (8-iso prostaglandin F2 alpha) was performed on plasma with the use of a commercial colorimetric kit (8-isoprostane EIA kit; Cayman Chemical Company, MI). The assay is based on competition between 8-isoprostane and 8-isoprostane acetylcholinesterase conjugate (8-isprostane tracer) with fixed attachment sites for specific serum antibody to 8-isoprostane. Because the concentration of the tracer is kept constant and the 8-isoprostane in the sample varies, the amount of tracer bound to serum antibody will be inversely proportional to the concentration of 8-isoprostane in the measuring plate. The product of the enzyme reaction was measured spectrophotometrically at 412 nm.[38]

Reducing ability of plasma

Total antioxidant capacity of the plasma of mothers was assessed by the ferric reducing ability of plasma (FRAP).[39] Briefly, the FRAP assay working reagent was prepared by mixing 300 mmol/L of acetate buffer (pH 3.6), 10 mmol/L of Tripyridil-s-triazine (TPTZ) solution, and 20 mmol/L of $FeCl_3 \times 6H_2O$ in a 10:1:1 ratio and by subsequent heating of the resultant mixture to 37°C. The reaction mixture was composed of 750 μL FRAP solution, 75 μL H_2O, and 25 μL samples and incubated in the dark at 25°C for 30 minutes; the absorbance was read at 593 nm. A standard curve that ranged from 50 μmol/L to 1.5 mmol/L of $FeSO_4$ was prepared for the quantitative determination of $FeSO_4$ as millimolar Fe^{2+} and $FeSO_4$ equivalents that was produced in the samples.[39]

Statistical analyses

All data were expressed as means ± SEM. Time of delivery was analyzed by a Watson-William test. Kolmogorov-Smirnov test was used to confirm normality of the data; comparisons were done by an unpaired *t*-test. Differences were accepted as significant at a probability value of ≤.05 (Prism 5.0; GraphPad Software Inc, La Jolla, CA).

Results

Melatonin antenatal administration induced changes in the normal development of pregnancy at chronic hypobaric hypoxia, increasing the length of pregnancy by 7.5% and shifting labor and delivery time from early morning deliveries to afternoon and evening events (MC, 5:38 ± 0:40 AM vs MM, 6:42 ± 1:06 PM; $P < .05$; Figure 1). This represents an average shifting time of approximately 12 hours. Additionally, melatonin caused a greater restriction of growth that manifested as a decreased birthweight, biparietal

FIGURE 1
Time of delivery

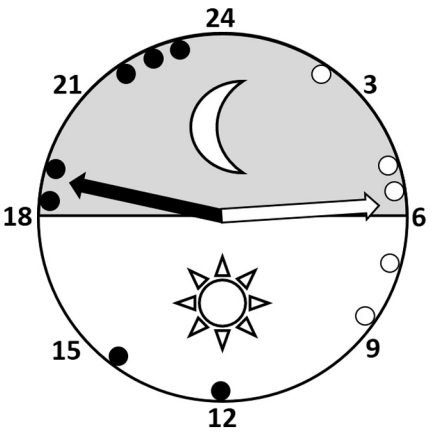

Diagram shows time of birth for all ewes that were included in the control group (*open circles/arrow*; n = 5) and melatonin-treated group (*closed circles/arrow*; n = 7). *Clock arrows* indicate the average time of delivery. Significant differences were found between groups (Watson-William test, $P < .02$).

MC, control group; *MM*, melatonin-treated group.

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diameter, crown-rump length, and abdominal diameter (Table). However, calculation of the ratios between biparietal diameter/birthweight and between crown-rump length/birthweight, evidenced similarity of the IUGR pattern between groups (Table).

The maternal plasma samples that were taken at 10:00 AM and 10:00 PM to determine melatonin, cortisol, FRAP, and 8-isoprostanes showed similar values between 120, 130, and 140 days of gestation at these hours. Therefore, we decided to average the results as daytime and nighttime samples.

Daytime samples showed similar values of melatonin concentration between groups (MC, 138.7 ± 31.5 pg*mL^{-1} vs MM, 187.5 ± 31.5 pg*mL^{-1}). In marked contrast, plasma melatonin raised 2-fold at nighttime in mothers who were treated with melatonin (MC, 654.2 ± 160.4 pg*mL^{-1} vs MM, 1481.0 ± 229.9 pg*mL^{-1}; $P < .05$; Figure 2A).

Maternal cortisol levels were similar between daytime and nighttime samples in control ewes. In contrast, melatonin induced increased cortisol levels in morning samples (MC, 1.422 ± 0.528 pg*mL^{-1} vs MM, 9.250 ± 1.366 pg*mL^{-1}; Figure 2B).

In addition, melatonin treatment increased the maternal plasma antioxidant capacity during daytime (MC, 311.8 ± 23.7 μmol/L vs MM, 484.9 ± 45.3 μmol/L; $P < .05$) and nighttime (MC, 420.4 ± 66.7 μmol/L vs MM, 616.2 ± 44.9 μmol/L; $P < .05$; Figure 3A). Moreover, melatonin administration clearly decreased plasma levels of 8-isoprostane at nighttime (MC, 16.12 ± 2.40 pg*mL^{-1} vs MM, 4.65 ± 0.40 pg*mL^{-1}; $P < .05$); there was no difference in daytime samples between groups (MC, 13.18 ± 2.3 pg*mL^{-1} vs MM, 8.74 ± 0.8 pg*mL^{-1}; Figure 3B).

Comment

This study shows for the first time the effects of an antenatal treatment with melatonin on gestation length and neonatal biometry in pregnancies that were exposed to chronic hypoxia at high altitude. Melatonin treatment markedly extended gestational length and significantly shifted the modal time of birth but decreased neonatal biometric parameters. Our results are contrary to most of the findings in animal models, in which melatonin treatment during pregnancy has not shown any detrimental effects on the offspring outcome.[11,12,14-17,40] Because birthweight and size are related closely to clinical neonatal outcomes, our findings highlight the need of further studies before translating melatonin to clinical practice, at least in gestations that may be affected by chronic hypoxia.

Delivery is influenced by several factors for both the mother and the term fetus. One of the signals that initiate labor is triggered by an increase in fetal cortisol secretion, decreased progesterone, and augmented primary prostaglandins.[41] Melatonin is able to inhibit cortisol secretion from the fetal adrenal cortex,[42] therefore delaying labor onset. Further, previous studies showed that melatonin inhibits the expression of 3β-hydroxysteroid dehydrogenase enzyme in the adrenal cortex, thereby decreasing corticotropin-induced cortisol synthesis.[42,43] Therefore, melatonin administration would selectively inhibit the fetal production of cortisol and may explain the increased length of gestation found in our studies.

Maternal cortisol increases maternal glycemia.[44] These effects may induce hyperglycemia and hyperinsulinemia for several days in the fetal lamb, which causes a sustained increase in umbilical glucose uptake and a further decrease in fetal arterial oxygen content.[45] In high altitude, this effect could decrease the O_2 availability even more for the fetus by impacting an already decreased fetal growth. Further, the glucose and oxygen

TABLE
Gestational and neonatal characteristics

Variable	Control group (n = 5)	Melatonin-treated group (n = 7)
Gestational length, d	149 ± 1	155 ± 1[a]
Birthweight, kg	3.56 ± 0.16	2.88 ± 0.22[a]
Biparietal diameter, mm	64.98 ± 1.53	55.50 ± 2.06[a]
Crown-rump length, cm	47.98 ± 1.82	37.98 ± 1.40[a]
Abdominal diameter, cm	38.70 ± 1.53	32.57 ± 1.71[a]
Biparietal diameter/birthweight, mm/kg	18.73 ± 1.24	19.86 ± 1.68
Crown-rump length/birthweight, cm/kg	13.48 ± 1.17	13.60 ± 1.17

Values are mean ± SEM.

[a] Significant differences by unpaired *t*-test ($P < .05$) vs the control group.

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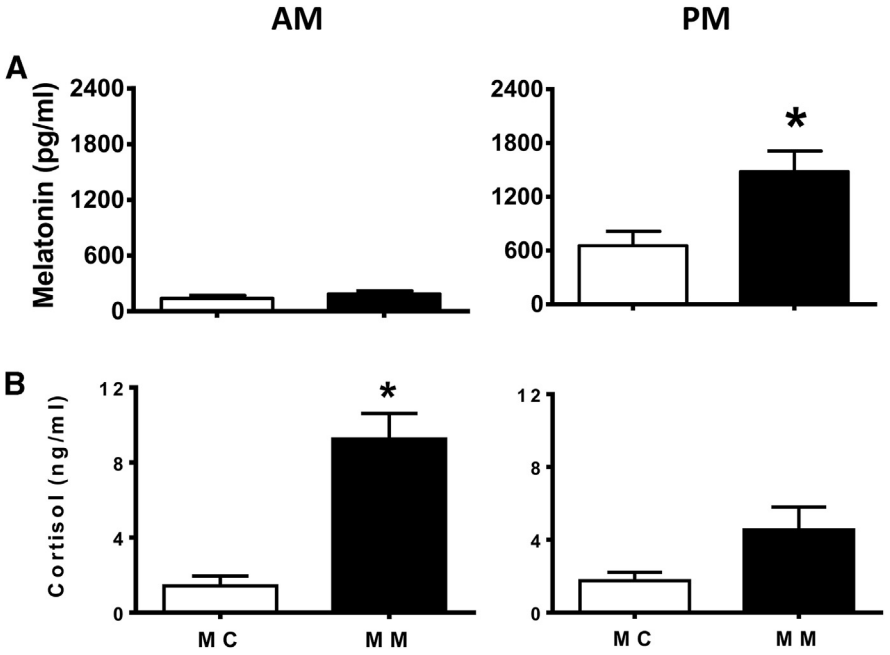
FIGURE 2
Melatonin and cortisol concentration in maternal plasma



Histograms of **A,** maternal melatonin and **B,** cortisol levels for the control (n = 5) and melatonin-treated (n = 7) pregnant ewes. Data are expressed as mean ± SEM for morning sampling (10:00 AM) and night sampling (10:00 PM). The *asterisks* indicate significant differences (unpaired *t*-test, $P < .05$) vs the control group.

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mismatch may alter the fetal metabolism and induce acidemia among other physiological alterations.[46] Clearly, a lower birthweight and the impaired metabolic outcome of the neonates might be programming the offspring to experience the development of diseases that manifest later in life.[44,47,48] All of these possible scenarios must be evaluated in further experiments.

Our study suggests that melatonin is regulating gestational length and time of parturition. In the light of these observations, we postulate that melatonin might be promoting progesterone synthesis, which is essential for pregnancy maintenance.[49,50] Further, melatonin may be decreasing glucocorticoid synthesis in the fetal adrenal gland.[42,51] The latter will impact decreased placental aromatase activity and 17α hydroxylase, both of which increase during the prepartum phase.[52,50] Additionally, melatonin is known to inhibit uterine contractility by decreasing prostaglandin synthesis.[53] Thus, melatonin may cause a marked fall in fetal plasma prostanoids, which may also be diminishing maturation and parturition signaling.[54] All or some of the aforementioned mechanisms might be delaying the parturition in the high-altitude sheep that were treated with melatonin and might also be translated in other species, such as humans. However, further studies are needed to evaluate the mechanisms involved in this model.

The enhanced growth restriction observed in newborn infants who were treated with prenatal melatonin is of concern. One of the possible mechanisms is the cardiovascular effects of melatonin. Although maternal blood pressure in pregnant ewes was not measured, 1 of the potential side-effects of maternal melatonin is hypotension,[55,56] and therefore uteroplacental decreased perfusion with eventual restriction in oxygen and nutrient delivery. In fact, there is a correlation between maternal falls in systemic arterial pressure during pregnancy and small babies.[57] In addition, we propose another possible explanation, based on broad redox status changes during night- and daytime in the developing fetus, which may increase the oxidative stress damage during daytime. The fact that melatonin acts as a potent scavenger[58] that is associated with a fast daytime clearance[59] may induce dramatic changes in the maternal and fetal antioxidant capacity with circadian pattern. Further, it has been shown that hypoxic lambs show a decreased messenger RNA expression for insulin-like growth factor (IGF)[60] that is associated with a marked decreased in fetal growth. This is usually associated with increased levels of the IGF binding protein-1, which in turn restricts the IGF-mediated fetal growth.[61-63] Therefore, the alterations of the IGF pathway is likely to be 1 of the determinant mechanisms of IUGR at high altitude. To the best of our knowledge, no studies have addressed the effects of melatonin on IGF in fetuses. However, in diverse adult tissues, it has been shown that melatonin may decrease IGF or its actions, which reduces growth rates.[64-66] Hence, here we proposed that melatonin might be decreasing fetal growth by IGF-related pathways. Further studies should focus on these mechanisms to address whether melatonin decreases IGF and further reduces fetal growth at high altitude.

Coherent and solid studies have suggested that melatonin has no adverse effects on prenatal survival, fetal body weight, or incidences of fetal malformations when given to pregnant animals in doses up to 200 mg/kg/d.[10,12,17,40] In fact, until now, there is no evidence that melatonin treatment has any effects in prenatal growth, either acute or chronically administered in normal and healthy pregnancies[40,67] by the exception of an increased mortality rate in pups from pregnant Wistar-Kyoto rats that were treated orally with melatonin.[68] In

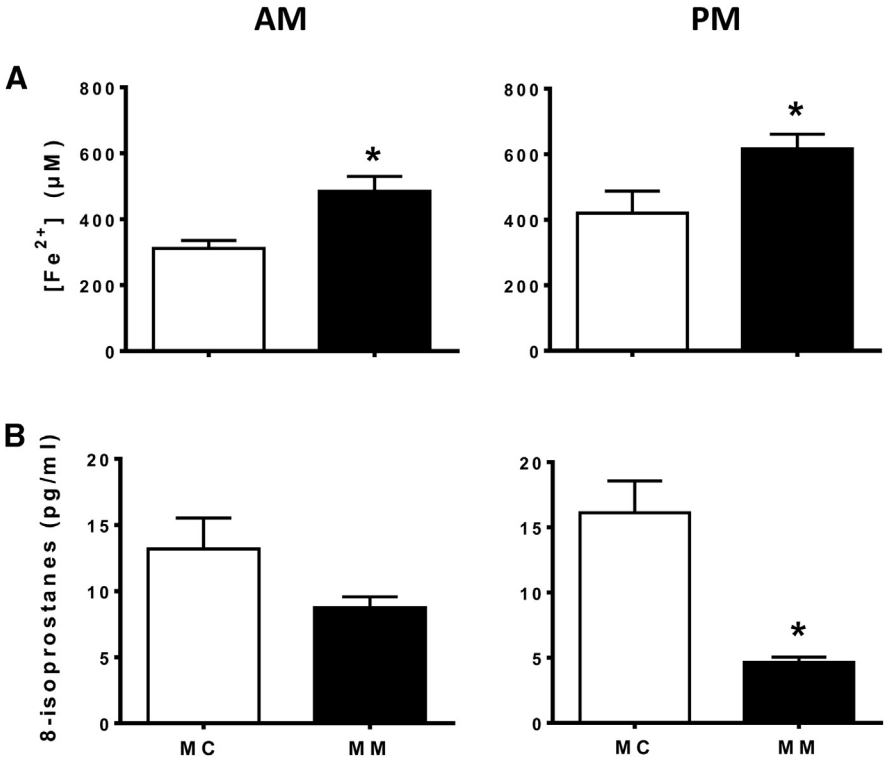
addition, a recently published study in chick embryos showed that melatonin did not recover IUGR that was induced by chronic hypoxia during incubation.[69]

Birthweight is a strong predictor of neonatal survival and morbidity[70]; therefore, the marked growth restriction that is induced by melatonin strongly suggests that the proposed therapy under chronic hypoxia is detrimental for the clinical outcome of the neonates.

The plasma redox capacity improved in mothers who were treated with melatonin. This was not only an expected finding because melatonin has potent scavenger activity but also can stimulate antioxidant enzymes under normal and oxidative stress conditions.[17,20,71] In fact, melatonin treatment during pregnancy restores the protein expression of Mn-SOD, catalase and glutathione peroxidase, with decreases in oxidative stress markers.[16,17,69] Further, a recent study showed the high effectiveness of melatonin in reversing the cardiovascular impairment that is induced by hypoxia.[69] All of the aforementioned studies suggest that melatonin could be a candidate for protection and/or treatment of cardiovascular dysfunctions that are induced by placental insufficiency. However, the IUGR condition was reverted partially only in some of them.[16,17]

In summary, scientific literature during the last decade has contributed solid evidence to consider melatonin as a potential treatment to prevent or treat IUGR that is associated with hypoxia and oxidative stress. This is based on its pleiotropic characteristics, which favor vasodilation and decreasing oxidative stress. Until now, melatonin has been considered as a highly effective antioxidant with few side-effects. Our results highlight the fact that caution should be taken when considering antenatal treatment with melatonin, and details in the diagnosis should be taken into account for the appropriate treatment. Taking attention to this study, the use of melatonin in pregnant women under chronic hypoxic conditions should be reevaluated, at least in a high-land population. This does not undermine a really provocative and important proposal of several research groups that are searching for melatonin benefits, providing an interesting platform from which more detailed experiments should clarify how and when melatonin therapy may be beneficial for complicated pregnancies.■

FIGURE 3
Antioxidant capacity and oxidative stress in maternal plasma



Histograms of **A,** maternal reducing capacity of plasma and the **B,** oxidative stress marker, 8-isoprostane for the control (n = 5) and melatonin-treated (n = 7) pregnant ewes. Data are expressed as mean ± SEM for morning sampling (10:00 AM) and night sampling (10:00 PM). The *asterisks* indicate significant differences (unpaired *t*-test, $P < .05$) vs the control group.

MC, control group; *MM*, melatonin-treated group.

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