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Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction—a phase I pilot clinical trial: study protocol

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ABSTRACT

Background: Fetal growth restriction complicates about 5% of pregnancies and is commonly caused by placental dysfunction. It is associated with increased risks of perinatal mortality and short-term and long-term morbidity, such as cerebral palsy. Chronic in utero hypoxaemia, inflammation and oxidative stress are likely culprits contributing to the long-term neurological sequelae of fetal growth restriction. In this regard, we propose that melatonin, a powerful antioxidant, might mitigate morbidity and/or mortality associated with fetal growth restriction. Melatonin has an excellent biosafety profile and crosses the placenta and blood–brain barrier. We present the protocol for a phase I clinical trial to investigate the efficacy of maternal oral melatonin administration in women with a pregnancy complicated by fetal growth restriction.

Methods and analysis: The proposed trial is a single-arm, open-label clinical trial involving 12 women. Severe, early onset fetal growth restriction will be diagnosed by an estimated fetal weight ≤10th centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal well-being, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then start melatonin treatment (4 mg) twice daily until birth. The primary outcomes are the levels of oxidative stress in the maternal and fetal circulation and placenta. Secondary outcomes are fetoplacental Doppler studies (uterine artery, umbilical artery middle cerebral artery and ductus venosus), fetal biometry, fetal biophysical profile and a composite determination of neonatal outcome. A historical cohort of gestational-matched fetal growth restriction and a healthy pregnancy cohort will be used as comparators.

Ethics and dissemination: Ethical approval has been obtained from Monash Health Human Research Ethics Committee B (HREC12133B). Data will be presented at international conferences and published in peer-reviewed journals.

Trial registration number: Clinical Trials, protocol registration system: NCT01695070.

Strengths and limitations of this study

- This pilot study has an appropriate design, and includes sufficient participants, to achieve measureable outcomes.
- Extensive previous human and animal studies form the basis for the design of this trial.
- This pilot study is the first of its kind in the world. Being a pilot study, this trial lacks randomisation and a placebo group, although comparator groups are included. If this trial is successful, the results will be used to inform future randomised controlled trials.

INTRODUCTION

Fetal growth restriction (FGR) defines the fetus that does not realise its normal growth potential. This significant pregnancy complication accounts for about 5% of all pregnancies.[1] FGR is most commonly caused by placental dysfunction.[2] FGR is strongly associated with increased risks of preterm birth, perinatal death, and, among survivors, significant cardiovascular and neurological morbidities, including cerebral palsy.[3–7]

State-of-the-art fetal surveillance techniques are important in the diagnosis and surveillance of the growth restricted fetus, and afford timely delivery. However, with the exception of antenatal glucocorticoids there are currently no treatments to reduce the short-term and long-term morbidities associated with FGR.[8] In FGR, injury to the developing fetal brain is likely to principally occur in utero, emphasising that, if the burden of neurological morbidity is to be reduced, then antenatal neuroprotective treatment, as opposed to postnatal therapy, will be necessary. Thus, future treatment should be directed at protecting fetal brain development and reducing the incidence of brain injury before birth rather than solely focusing on care in the nursery.

Although many in utero events can perturb normal fetal brain development, it is thought that chronic feto-placental hypoxaemia and oxidative stress are the likely key mechanisms initiating pathways leading to brain injury.[9] In placental dysfunction, the placenta is exposed to recurrent ischaemia-reperfusion injury, ultimately leading to decreased placental oxygen transfer capacity. Consequently, the placenta cannot adequately meet the growing metabolic needs of the fetus, which in turn becomes progressively hypoxaemic. Compensatory fetal mechanisms attempt to maintain adequate oxygenation, particularly of the heart and brain. Nevertheless, these eventually fail and a complex cascade of metabolic events is initiated that involves the generation of nitrogen and oxygen free radicals.[10]

The central nervous system, and in particular the fetal brain, is particularly susceptible to free radical damage.[11] Low antioxidant defenses and abundant iron within the fetal brain render it vulnerable to oxidative stress. Furthermore, the fetal brain is rich in membrane lipids that are sensitive to free radical attack. Indeed, lipid peroxidation plays an important role in neuronal and white matter damage directly and indirectly. Byproducts of lipid peroxidation can trigger vasoconstriction,[12] and are cytotoxic, mutagenic and teratogenic.[13–15] MRI of preterm infants with white matter injury shows significantly higher levels of protein and lipid peroxidation.[16] Furthermore, in an autopsy study on human brain tissue, activated microglia, significant protein nitration, lipid peroxidation and cell injury were detected in premyelinating oligodendrocytes in babies diagnosed with periventricular leukomalacia.[17]

Melatonin (5-methoxy-*N*-acetyltryptamine) is an endogenous lipid-soluble hormone, predominantly produced in the pineal gland that is best recognised for its role in providing circadian and seasonal timing cues. It is also a strong antioxidant. It effectively scavenges free radicals, induces endogenous antioxidant enzymes, such as glutathione peroxidase and glutathione reductase[18] and inhibits the pro-oxidative enzyme nitric oxide synthase.[19] Melatonin also stabilises cell membranes, enhancing their resistance to oxidative damage.[20] In vitro, melatonin treatment protects the villous trophoblast against hypoxia-reperfusion-induced oxidative stress and apoptosis.[21] Owing to its strong antioxidant capacity and its ability to cross the human placenta[22] and blood–brain barrier,[23] melatonin is of particular interest as a candidate antenatal neuroprotectant. The neuroprotective potential of melatonin is augmented by the fact that melatonin inhibits norepinephrine-induced middle cerebral artery constriction[24] and induces umbilical vasodilation[25] in ovine models of FGR. In animal models, we have shown that melatonin treatment before and during transient severe fetal asphyxia prevented the formation of hydroxyl radicals within the fetal brain,[26] reduced lipid peroxidation and cell death and stabilised the blood–brain barrier.[27] Administration of melatonin at the time of induced hypoxia in the fetal sheep brain also reduced inflammation and cell death.[28] Using an established ovine model of FGR,[29] we have now shown that maternal administration of melatonin reduced fetal hypoxia, improved neurodevelopment and decreased brain injury and oxidative stress in newborn lambs (SL Miller, unpublished data 2013).

Melatonin treatment during human pregnancy has been studied for a large range of conditions and at different times during gestation. Predominantly, melatonin has been studied in assisted reproductive technology, aiming to improve oocyte quality and pregnancy rates following in vitro fertilisation (IVF). Melatonin administration, started prior to IVF-cycles and continued during pregnancy, was associated with modestly improved pregnancy outcomes.[30–32] Importantly, all babies who were born from melatonin-treated pregnancies were born healthy and with no congenital abnormalities (V Unfer, personal communication, 2012). Furthermore, maternal treatment with melatonin significantly boosts placental antioxidant enzyme gene expression.[22]

Except for its known, benign effects on sleep dynamics, melatonin does not exert acute pharmacological effects on the nervous or vascular system. A median lethal dose in mice could not be calculated because an increased mortality rate was not observed, even following administration of extremely high doses of up to 800 mg/kg melatonin.[33] Long-term nightly oral treatment with 75 mg melatonin in 1400 non-pregnant women did not cause any serious adverse effects.[34] To our knowledge, no maternal and/or developmental toxicity effects, due to melatonin treatment, have been reported. The maternal no adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) were established at 100 and 200 mg/kg/day, respectively, for maternally administered melatonin in pregnant rats. The developmental toxicity NOAEL was $\geq$ 200 mg/kg/day.[35]

Rationale

FGR is a serious pregnancy complication, affecting up to 5% of pregnancies. It is associated with significant fetal and neonatal mortality and morbidity, including neurological impairment. Even though modern obstetrics allow for careful fetal monitoring, there are neither effective therapies for FGR nor established options for antenatal neuroprotection in FGR. Numerous animal studies have established the use of melatonin as an effective antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this trial, we aim to explore, for the first time, whether melatonin is an effective antioxidant in human FGR.

Aims

We will establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by FGR.

Specific aims are:

1. To determine the effect of daily maternal oral melatonin on markers of oxidative stress in the placenta,

and maternal and fetal circulations in pregnancies affected by the early onset FGR.

2. To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by the early onset FGR.

3. To determine the effect of daily maternal oral melatonin on brain injury and neurodevelopment in growth-restricted newborn babies.

METHODS AND ANALYSIS
Study design
Phase I single-arm, open label clinical trial, with historic cohorts of FGR and healthy pregnancies as comparators.

Subjects
Twelve women (pregnancies) with FGR will be recruited and studied to completion of the trial. A historical cohort of gestational-matched FGR and a healthy pregnancy cohort will be used as comparators.

Patient eligibility
Pregnant women with a singleton growth-restricted fetus between 23^{+0} and 34^{+0} weeks (confirmed gestational age). FGR will be defined as a sonographic-estimated fetal weight at or below the 10th centile for gestational age in combination with at least one abnormal fetoplacental Doppler study of the uterine artery (bilateral uterine artery notching or unilateral uterine artery notching on the ipsilateral side of the placenta), umbilical artery (systolic : diastolic ratio ≥95th centile or absent, or, reversed end-diastolic flow), middle cerebral artery (pulsatility index ≤5th centile) or ductus venosus (abnormal A wave and/or pulsatility index ≥95th centile). For consenting purposes, mothers must be 18 years or older and have a basic understanding of English. Women with an unconfirmed pregnancy duration, a multiple pregnancy or a pregnancy affected by maternal or fetal infection or with a fetus with a chromosomal or major structural anomaly or non-placental FGR will be excluded from this trial.

Recruitment
Patients will be recruited from the maternity services of Monash Health and Jessie McPherson Private Hospital, Melbourne, Australia. The study will be staffed with a clinical research team, who will inform potential participants about the aims, methods and potential risks and benefits of this study. Participants will be informed that their participation is voluntary and that they may withdraw consent to participate at any time during the study, and for any reason, and that choosing not to participate or withdraw will not affect their care. An information form about the trial and opportunities to ask questions will be offered to the woman and any other person(s) she chooses. Women will be recruited to the study following written informed consent.

Trial treatment
Following recruitment, and until delivery, women will receive oral melatonin prolonged release (Circadin, Neurim Pharmaceuticals), 4 mg twice daily (a total daily dose of 8 mg) approximately 12 h apart. This dose and regimen are based on the results from our own experimental animal research, previously published animal research and clinical trials.

In our experimental FGR study, a maternal, cumulative, intravenous dose of 6 mg/day was administered. The human equivalent dose (HED) was calculated using the FDA-dose-conversion algorithm[36] and the average Australian height and weight. This resulted in a dose between 7.3 (based on prepregnancy weight) and 8.1 mg/day (based on end-pregnancy weight). As Circadin is only available in 2 mg tablets, the chosen dose of this study is 8 mg/day.

This dose of melatonin is lower than the doses used in several clinical studies in neonates. These trials evaluated melatonin as an antioxidant treatment in newborns with birth asphyxia and other conditions associated with increased oxidative stress. Melatonin was administered orally in doses up to 80 mg/day in these trials without any toxicity.[37–41] In clinical trials using melatonin to improve assisted reproductive technology, melatonin was administered orally in doses of 3 mg/day before and during pregnancy.[30 32 42] Melatonin treatment, started prior to conception and continued until birth, did not adversely affect pregnancy outcome and fetal or neonatal health (V Unfer, personal communication, 2012). The dose in this trial is higher than previously used doses in human pregnancy but still falls well below the maternal and fetal NOAEL (HED 16 and 32 mg/kg respectively) and maternal LOAEL (HED 32 mg/kg).

Melatonin will be administered orally as 4 mg given twice daily. This dosage regimen is chosen because the terminal half-life of Circadin is 3.5–4 h. In FGR, increased levels of oxidative stress likely occur due to the continuous placental generation of reactive oxygen species. By administering melatonin twice daily, we aim to provide a sustained increase in the maternal and fetal serum. To minimise daytime sleepiness and severe disturbances of circadian rhythms, melatonin will be given early in the morning and at night.

Sample collection
Prior to starting melatonin, a maternal blood sample will be taken and ultrasound and Doppler measurements will be obtained. This will be repeated once per week until birth. Immediately prior to birth, a maternal blood sample will be obtained. At the time of birth, samples of the amniotic fluid, umbilical cord blood and placenta will be collected. Clinical details of pregnancy, including medication and (multi) vitamin use, delivery and neonatal outcomes will be recorded in prespecified case record forms.

All blood samples will be collected in serum tubes and blood RNA tubes, allowing for measurement of markers

of oxidative stress and gene expression studies, respectively. Serum tubes will be centrifuged (4400 g, 15 min). The serum will be collected and transferred into collection tubes. The collection tubes will be stored in a −80°C until further processing.

Amniotic fluid will be collected during caesarean section deliveries. The myometrium will be bisected sharply and distinctly from the amnion and chorion. The amniotic fluid will be aspirated using a sterile syringe and 18-gauge drawing-up needle. Samples visibly contaminated with blood will be discarded. Within 30 min of the sample collection, specimens will be transferred to a laboratory. The samples will be centrifuged (1000 g, 15 min) and the supernatant will be collected, transferred into sterile collection tubes and snap frozen. Specimens will be stored at −80°C until further analysis.

Placentas will be obtained at the time of delivery and placed in a sterile tray. The area at the midpoint of the longest distance between the cord insertion and the edge of the placental disc will be sampled. Six full thickness placental samples will be cut out using sterile scissors. The placental tissue samples will be removed from the membranes using sterile forceps and scissors. The placental tissue samples will then be washed in a sterile container using ice cold, sterile phosphate-buffered saline. Two tissue samples will be fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. Two tissue samples will be placed in sterile tubes containing RNA later for 24 h at 4°C. After immersion in RNA later, these two samples will be transferred into two sterile cryovials, snap frozen in liquid nitrogen and stored at −80°C until further processing for gene expression studies. The remaining tissue samples will be placed into sterile cryovials and snap frozen in liquid nitrogen. Samples will be stored in a −80°C freezer until further processing to measure levels of oxidative stress and melatonin.

Outcome measures

The primary outcome will be the level of oxidative stress in the placenta and in the maternal (before and during treatment and at delivery) and fetal (umbilical artery and vein at delivery) circulations, as reflected by the measurement of malondialdehyde, 8-isoprostane, total antioxidant capacity and superoxide dismutase. Levels of melatonin will be measured in maternal and fetal blood and in the placenta. Gene expression of pro-oxidant and antioxidant enzymes, melatonin synthesising enzymes, melatonin receptors and clock genes will be measured in the placenta and in the maternal and fetal blood.

The secondary outcome measures will include clinical parameters about the pregnancy and its duration, fetal and neonatal well-being, mortality and morbidity (boxes 1–4).

Follow-up of women and infants

Participants will be followed weekly for the duration of the trial. The routine clinical care of participants,

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Box 1   Ultrasound and Doppler measurements

Doppler and ultrasound measurements: at recruitment and then weekly until delivery.
   Doppler velocimetry: umbilical artery, middle cerebral artery, ductus venosus and maternal uterine arteries
   Biometry (fortnightly)
   Amniotic fluid index
   Fetal behaviour: heart rate, tone, breathing, gross body movements

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Box 2   Maternal morbidity

Markers of maternal morbidity: at recruitment and at least weekly until delivery
   Blood pressure
   Complete blood count
   Liver function tests (aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase and bilirubin)
   Renal function tests (urea, creatinine and uric acid)

Box 3   Pregnancy end-points

Pregnancy outcomes:
   Gestational age at birth
   Diagnosis to delivery interval
   Abnormal cardiotocogram
   Indication for delivery (if not spontaneous)
   Mode of birth
   Labour analgesia/anaesthesia
   Duration of labour stages
   Duration of membrane rupture to birth
   Used medication such as antihypertensives, magnesium sulfate and corticosteroids
   Use of (multi) vitamins and dietary supplements

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including ultrasound scans, will not be affected by participation in the study. During the weekly follow-up, fetal and maternal well-being will be assessed and women will discuss any issues that they have experienced. Medication will be supplied weekly to meet the requirements for the following week. Women will be asked to keep a medication and symptom diary (drowsiness,

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Box 4   Neonatal outcomes

Neonatal outcomes:
   Sex
   Apgar scores (1, 5 and 10 min)
   Birth weight
   Umbilical cord S100B
   Head circumference at birth
   Composite neonatal outcome: admission to neonatal intensive care unit, duration of admission, need for and duration of respiratory support, intraventricular haemorrhage, necrotising enterocolitis, abnormal neurology, mortality prior to discharge

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sleepiness, general physical well-being, time of melatonin intake), which will be reviewed weekly by the principal investigator. Melatonin treatment will cease as soon as birth has occurred. Postnatal clinical care of the women will be the responsibility of the attending obstetric team. The principal investigator will assess the clinical condition of participants daily during the first 3 days postpartum, to ensure that no problems become apparent in response to withdrawal from treatment.

Clinical data will be obtained from neonates who require admission to the neonatal intensive care unit. Long-term follow-up using neurodevelopmental examination and questionnaires and MRI are desirable but will depend on funding.

Sample size and statistical analysis of data

This study is a proof-of-principle phase I trial. As such, there are no current data available to undertake a formal power calculation to determine the sample size. If successful, this exploratory study will provide the measures needed to perform the power calculations required for the planned phase II randomised controlled trial. This trial will provide data for the establishment of the use of maternal melatonin treatment in FGR.

The results obtained in this trial will be compared to those from historic cohorts of untreated FGR and healthy pregnancies, which we have been collecting since 2011, using the same protocol.

Biomarkers of oxidative stress in the maternal blood samples will be analysed using a repeated measures analysis of variance (ANOVA), including the baseline value as a covariate. It is assumed that the effect of treatment with melatonin on levels of oxidative stress will not depend on duration of treatment. However, secondary analysis will be performed to determine the effect of treatment duration on biomarkers of oxidative stress. If the treatment duration significantly impacts on the effect of melatonin on the oxidative stress status, this will be further investigated using the least-square measure. Data will be plotted as mean scores over time.

Biomarkers of oxidative stress in the maternal blood samples will be analysed using a repeated measures ANOVA, including the baseline value as a covariate. Levels of oxidative stress in the maternal blood sample obtained immediately prior to birth will be compared with samples derived from the historic cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will be compared with data obtained from the previously described historic cohorts. Clinical data regarding the maternal well-being, pregnancy outcome and neonatal outcome will be compared with the same cohorts. These data will be analysed using a one-way ANOVA.

A p value ≤ 0.05 is considered to be statistically significant.

Adverse events and safety monitoring

During the treatment period, clinical care will remain unchanged and will be the responsibility of uninvolved medical staff. The principal investigator will monitor all participants weekly, take a brief medical history, measure blood pressure and interpret ultrasound scan results (obtained by uninvolved staff) to ensure melatonin treatment can be safely continued. A weekly blood sample will be obtained to perform a full blood examination, assess liver and renal function and assess C reactive protein. For the duration of the trial, the principal investigator will be contactable by telephone at all times.

Any adverse medical events reported by the patient will be recorded in the patients' medical records at each visit. Serious adverse events occurring after initiation of the trial will be reported to the Monash Health Human Research Ethics Committee, Therapeutics Committee and Therapeutic Goods Administration of Australia's Office of Scientific Evaluation within 72 h.

Trial discontinuation or modification

The participants will be able to end participation at their own request at any time. Participants will be withdrawn from the trial if they or their fetus suffer from an unexpected serious adverse event. Worsening of fetoplacental Doppler velocimetry or fetal growth is within the natural history of FGR and is not part of the discontinuation criteria.

DISCUSSION

The use of melatonin as an antenatal antioxidant therapy in FGR has been proposed as a promising and safe option to protect the fetal brain from injury. This trial is essential to confirm the antioxidant capacity and safety of melatonin treatment. If this study confirms that maternal melatonin can decrease maternal, fetal and/or placental levels of oxidative stress, we will proceed to a phase II randomised controlled trial to assess the effect of maternal melatonin treatment on brain injury and neurodevelopment. In addition, melatonin treatment could also potentially be used as an antenatal neuroprotectant in other compromised pregnancies or in threatened preterm birth to protect the mother and/or fetus. Finally, if melatonin treatment indeed strengthens antioxidant defenses during pregnancy, it might be a suitable new strategy in the management of pre-eclampsia. A phase I trial to investigate the clinical and biochemical benefit of melatonin treatment in pre-eclampsia has been initiated.[43]

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