BMJ open

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

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-004141
Article Type:	Protocol
Date Submitted by the Author:	27-Sep-2013
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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Obstetrics and gynaecology
Keywords:	Fetal medicine < OBSTETRICS, Maternal medicine < OBSTETRICS, OBSTETRICS

Title: 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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Keywords: Fetal growth restriction, melatonin, clinical trial, oxidative stress, antenatal neuroprotection.

Word count: 2, 406.

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- and long-term morbidity, such as cerebral palsy. Chronic *in utero* hypoxiaemia, 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 estimated fetal weight ≤10th centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal wellbeing, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then commence melatonin treatment (4mg) 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, 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.

ARTICLE SUMMARY

Article focus:

- This trial aims to establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.
- Specific aims are:
    - To determine the effect of daily maternal oral melatonin on levels of oxidative stress in the placenta and in the maternal and fetal circulation in pregnancies affected by early onset fetal growth restriction.
    - To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
    - To determine the effect of daily maternal oral melatonin on brain injury and neurologic development in growth restricted newborn babies.

Key messages:

- Fetal growth restriction is a serious pregnancy complication and is associated with significant fetal and neonatal morbidity and mortality. Fetal brain injury is common in fetal growth restriction.

- Brain injury in survivors of fetal growth restriction is likely to originate antenatally as a consequence of oxidative stress.
- There are currently no established treatments for fetal growth restriction or for neuroprotection in fetal growth restriction.
- Numerous animal studies have established the use of melatonin as an antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this study we aim to explore whether melatonin is an effective antioxidant in the treatment of human fetal growth restriction.

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, results will be used to inform future randomized 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 percent 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- and long-term morbidities associated with FGR(8). In FGR, injury to the developing fetal brain is likely to principally occur *in utero*, emphasizing 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 fetoplacental 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. Further, 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). Magnetic resonance imaging of preterm infants with white matter injury show 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 all 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 recognized 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 stabilizes cell membranes, enhancing their resistance to oxidative

damage(20). Due to its strong antioxidant capacity and its ability to cross the human placenta(21) and blood-brain barrier(22), 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(23) and induces umbilical vasodilation(24) in ovine models of fetal growth restriction. We have shown that melatonin treatment before and during transient severe fetal asphyxia prevented the formation of hydroxyl radicals within the fetal brain(25), reduced lipid peroxidation and cell death and stabilized the blood-brain-barrier(26). Administration of melatonin at the time of induced hypoxia in the fetal sheep brain also reduced inflammation and cell death(27). Using an established ovine model of fetal growth restriction(28), 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* fertilization (IVF). Melatonin administration, commenced prior to IVF-cycles and continued during pregnancy, was associated with modestly improved pregnancy outcomes(29–31). Importantly, all babies that 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(21).

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 extremely high doses of up to 800mg/kg melatonin(32). Long term nightly oral treatment with 75mg melatonin in 1400 non-pregnant women did not cause any serious adverse effects(33). 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 were established at 100 and 200mg/kg/day, respectively, for maternally administered melatonin in pregnant rats. The developmental toxicity NOAEL was ≥ 200mg/kg/day(34).

Rationale: Fetal growth restriction 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 allows for careful fetal monitoring, there are neither effective therapies for fetal growth restriction nor established options for antenatal neuroprotection in fetal growth restriction. 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 fetal growth restriction.

Aims: We will establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.

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 early onset fetal growth restriction.
2. To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
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 fetal growth restriction will be recruited and studied to completion of the trial. A historical cohort of gestational matched fetal growth restriction and a healthy pregnancy cohort will be used as comparators.

Patient eligibility: Pregnant women with a singleton growth restricted fetus at less than 34 weeks. Fetal growth restriction 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, umbilical artery, middle cerebral artery or ductus venosus. Women with 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 fetal growth restriction will be excluded from this trial.

Recruitment, interventions and procedures: 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. Subjects 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. Clinical details of pregnancy, delivery and neonatal outcomes will be recorded in pre-specified case record forms.

Following recruitment, and until delivery, women will receive oral melatonin (Circadin®, Neurim Pharmaceuticals), 4mg twice daily (a total daily dose of 8mg) approximately 12 hours apart. 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 data regarding maternal wellbeing, pregnancy and fetal and neonatal outcomes will be recorded as the trial proceeds.

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. Serum levels of melatonin will be measured in maternal and fetal blood.

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

Table 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

Table 2: Maternal morbidity.

Markers of maternal morbidity: at recruitment and at least weekly until delivery.
Blood pressure
Complete blood count
Liver function tests (AST, ALT, GTT, bilirubin)
Renal function tests (urea, creatinine)

Table 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
Use of antihypertensives
Use of magnesium sulphate
Use of corticosteroids

Table 4: Neonatal outcomes.

Neonatal outcomes:
Sex
Apgar scores (1, 5, 10 minutes)
Birth weight
Cord artery 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, necrotizing enterocolitis, abnormal neurology,

mortality prior to discharge

Follow up of women and infants: Participants will be followed weekly for the duration of the trial. The routine clinical care of participants, including ultrasound scans, will not be affected by participation in the study. During the weekly follow-up, fetal and maternal wellbeing 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, 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 three days post-partum, 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 scans, 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 randomized-controlled trial. This trial will provide data for the establishment of the use of maternal melatonin treatment in fetal growth restriction.

The results obtained in this trial will be compared to historic cohorts of untreated fetal growth restriction 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, 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 analysis of variance, including the baseline value as a covariate. Levels of oxidative stress in the maternal blood sample obtained immediately prior to birth will be compared to samples derived from the historic cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will be compared to data obtained from the previously described historic cohorts. Clinical data regarding maternal wellbeing, pregnancy outcome and neonatal outcome will be compared to 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 hours.

Trial discontinuation or modification

The participants can 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 are within the natural history of fetal growth restriction and are not part of the discontinuation criteria.

Ethics and dissemination: This study has been approved by the Monash Health Human Research Ethics Committee B (HREC 12133B). Data will be presented at relevant conferences and published in peer-reviewed journals.

DISCUSSION

The use of melatonin as an antenatal antioxidant therapy in fetal growth restriction 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 randomized-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 study to study the clinical and biochemical benefit of melatonin treatment in preeclampsia has been initiated(35).

AUTHORS' CONTRIBUTIONS

NOA: research, contribution of original material, editing and approval of final manuscript. SLM, GJ, EMW: editing and approval of final manuscript.

FUNDING STATEMENT

This work was supported by the 5Point Foundation.

COMPETING INTERESTS

NOA is funded by a Monash Graduate Scholarship and a Monash International Postgraduate Research Scholarship.

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BMJ open

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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-004141.R1
Article Type:	Protocol
Date Submitted by the Author:	20-Nov-2013
Complete List of Authors:	Alers, Nicole; Monash University, The Ritchie Centre, Monash Institute of Medical Research; Monash University, Department of Obstetrics and Gynaecology Jenkin, Graham; Monash University, The Ritchie Centre, Monash Institute of Medical Research; Monash University, Department of Obstetrics and Gynaecology Miller, Suzanne; Monash University, The Ritchie Centre, Monash Institute of Medical Research; Monash University, Department of Obstetrics and Gynaecology Wallace, Euan; Monash University, The Ritchie Centre, Monash Institute of Medical Research; Monash University, Obstetrics and Gynaecology
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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- 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 estimated fetal weight ≤10th centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal wellbeing, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then commence melatonin treatment (4mg) 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, middle cerebral artery, and ductus venosus), fetal biometry, fetal biophysical profile and a composite determination

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

Key messages:

- Fetal growth restriction is a serious pregnancy complication and is associated with significant fetal and neonatal morbidity and mortality. Fetal brain injury is common in fetal growth restriction.

- Brain injury in survivors of fetal growth restriction is likely to originate antenatally as a consequence of oxidative stress.
- There are currently no established treatments for fetal growth restriction or for neuroprotection in fetal growth restriction.
- Numerous animal studies have established the use of melatonin as an antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this study we aim to explore whether melatonin is an effective antioxidant in the treatment of human fetal growth restriction.

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, results will be used to inform future randomized 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 percent 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- and long-term morbidities associated with FGR(8). In FGR, injury to the developing fetal brain is likely to principally occur *in utero*, emphasizing 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 fetoplacental 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. Further, 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). Magnetic resonance imaging of preterm infants with white matter injury show 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 all 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 recognized 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 stabilizes 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). Due 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 fetal growth restriction. 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 stabilized 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 fetal growth restriction(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* fertilization (IVF). Melatonin administration, commenced prior to IVF-cycles and continued during pregnancy, was associated with modestly improved pregnancy outcomes(30–32).

Importantly, all babies that 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 extremely high doses of up to 800mg/kg melatonin(33). Long term nightly oral treatment with 75mg 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 200mg/kg/day, respectively, for maternally administered melatonin in pregnant rats. The developmental toxicity NOAEL was ≥ 200mg/kg/day(35).

Rationale: Fetal growth restriction 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 allows for careful fetal monitoring, there are neither effective therapies for fetal growth restriction nor established options for antenatal neuroprotection in fetal growth restriction. 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 fetal growth restriction.

Aims: We will establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.

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 early onset fetal growth restriction.
2. To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
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 fetal growth restriction will be recruited and studied to completion of the trial. A historical cohort of gestational matched fetal growth restriction 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). Fetal growth restriction will be defined as a sonographic estimated fetal weight at or below the 10^{th} 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 $\geq 95^{th}$ centile or absent, or, reversed end-diastolic flow), middle cerebral artery (pulsatility index $\leq 5^{th}$ centile) or ductus venosus (abnormal A wave and/or pulsatility index $\geq 95^{th}$ 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 fetal growth restriction 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. Subjects 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), 4mg twice daily (a total daily dose of 8mg) approximately 12 hours apart. This dose and regimen are based on 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 6.0mg/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 7.3mg/day (based on pre-pregnancy weight) and 8.1mg/day (based on end-pregnancy weight). As Circadin® is only available in 2mg tablets, the chosen dose for this study is 8mg per day.

This dose of melatonin is lower than the used doses 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 80mg/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 3mg/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 32mg/kg respectively) and maternal LOAEL (HED 32mg/kg).

Melatonin will be administered orally as 4mg given twice per day. This dosage regimen is chosen because the terminal half-life of Circadin® is 3.5 to 4 hours. In fetal growth restriction, increased levels of oxidative stress likely occur due to continuous placental generation of reactive oxygen species. By administering melatonin twice daily, we aim to provide a sustained increase in maternal and fetal serum. To minimize 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 pre-specified 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 (4400g, 15 minutes). 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 off. Within 30 minutes of the sample collection, specimens will be transferred to a laboratory. The samples will be centrifuged (1000g, 15 minutes) 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 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 hours 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- and antioxidant enzymes, melatonin synthesizing 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 wellbeing, mortality and morbidity (Tables 1-4).

Table 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

Table 2: Maternal morbidity.

Markers of maternal morbidity: at recruitment and at least weekly until delivery.
Blood pressure
Complete blood count
Liver function tests (AST, ALT, GTT, bilirubin)
Renal function tests (urea, creatinine, uric acid)

Table 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 sulphate and corticosteroids.
Use of (multi) vitamins and dietary supplements

Table 4: Neonatal outcomes.

Neonatal outcomes:
Sex
Apgar scores (1, 5, 10 minutes)
Birth weight
Cord artery 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, necrotizing enterocolitis, abnormal neurology, mortality prior to discharge

Follow up of women and infants: Participants will be followed weekly for the duration of the trial. The routine clinical care of participants, including ultrasound scans, will not be affected by participation in the study. During the weekly follow-up, fetal and maternal wellbeing 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, 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 three days post-partum, 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 scans, 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 randomized-controlled trial. This trial will provide data for the establishment of the use of maternal melatonin treatment in fetal growth restriction.

The results obtained in this trial will be compared to historic cohorts of untreated fetal growth restriction 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, 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 analysis of variance, including the baseline value as a covariate. Levels of oxidative stress in the maternal blood sample obtained immediately prior to birth will be compared to samples derived from the historic cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will be compared to data obtained from the previously described historic cohorts. Clinical data regarding maternal wellbeing, pregnancy outcome and neonatal

outcome will be compared to 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 hours.

Trial discontinuation or modification

The participants can 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 are within the natural history of fetal growth restriction and are not part of the discontinuation criteria.

Ethics and dissemination: This study has been approved by the Monash Health Human Research Ethics Committee B (HREC 12133B). Data will be presented at relevant conferences and published in peer-reviewed journals.

DISCUSSION

The use of melatonin as an antenatal antioxidant therapy in fetal growth restriction 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 randomized-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 study to study the clinical and biochemical benefit of melatonin treatment in preeclampsia has been initiated.

AUTHORS' CONTRIBUTIONS

NOA: research, contribution of original material, editing and approval of final manuscript. SLM, GJ, EMW: editing and approval of final manuscript.

FUNDING STATEMENT

This work was supported by the 5Point Foundation.

COMPETING INTERESTS

NOA is funded by a Monash Graduate Scholarship and a Monash International Postgraduate Research Scholarship.

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Title: 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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Keywords: Fetal growth restriction, melatonin, clinical trial, oxidative stress, antenatal neuroprotection.

Word count: 3143.

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- 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 estimated fetal weight ≤10th centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal wellbeing, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then commence melatonin treatment (4mg) 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, 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.

ARTICLE SUMMARY

Article focus:

- This trial aims to establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.
- Specific aims are:
    - To determine the effect of daily maternal oral melatonin on levels of oxidative stress in the placenta and in the maternal and fetal circulation in pregnancies affected by early onset fetal growth restriction.
    - To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
    - To determine the effect of daily maternal oral melatonin on brain injury and neurologic development in growth restricted newborn babies.

Key messages:

- Fetal growth restriction is a serious pregnancy complication and is associated with significant fetal and neonatal morbidity and mortality. Fetal brain injury is common in fetal growth restriction.

- Brain injury in survivors of fetal growth restriction is likely to originate antenatally as a consequence of oxidative stress.
- There are currently no established treatments for fetal growth restriction or for neuroprotection in fetal growth restriction.
- Numerous animal studies have established the use of melatonin as an antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this study we aim to explore whether melatonin is an effective antioxidant in the treatment of human fetal growth restriction.

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, results will be used to inform future randomized 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 percent 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- and long-term morbidities associated with FGR(8). In FGR, injury to the developing fetal brain is likely to principally occur *in utero*, emphasizing 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 fetoplacental 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. Further, 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). Magnetic resonance imaging of preterm infants with white matter injury show 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 all 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 recognized 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 stabilizes 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). Due 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 fetal growth restriction. 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 stabilized 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 fetal growth restriction(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* fertilization (IVF). Melatonin administration, commenced prior to IVF-cycles and continued during pregnancy, was associated with modestly improved pregnancy outcomes(30–32). Importantly, all babies that 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 extremely high doses of up to 800mg/kg melatonin(33). Long term nightly oral treatment with 75mg 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 200mg/kg/day, respectively, for maternally administered melatonin in pregnant rats. The developmental toxicity NOAEL was ≥ 200mg/kg/day(35).

Rationale: Fetal growth restriction 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 allows for careful fetal monitoring, there are neither effective therapies for fetal growth restriction nor established options for antenatal neuroprotection in fetal growth restriction. 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 fetal growth restriction.

Aims: We will establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.

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 early onset fetal growth restriction.
2. To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
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 fetal growth restriction will be recruited and studied to completion of the trial. A historical cohort of gestational matched fetal growth restriction 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). Fetal growth restriction 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 fetal growth restriction 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. Subjects 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), 4mg twice daily (a total daily dose of 8mg) approximately 12 hours apart. This dose and regimen are based on 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 6.0mg/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 7.3mg/day (based on pre-pregnancy weight) and 8.1mg/day (based on end-pregnancy weight). As Circadin® is only available in 2mg tablets, the chosen dose for this study is 8mg per day.

This dose of melatonin is lower than the used doses 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 80mg/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 3mg/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 32mg/kg respectively) and maternal LOAEL (HED 32mg/kg).

Melatonin will be administered orally as 4mg given twice per day. This dosage regimen is chosen because the terminal half-life of Circadin® is 3.5 to 4 hours. In fetal growth restriction, increased levels of oxidative stress likely occur due to continuous placental generation of reactive oxygen species. By administering melatonin twice daily, we aim to provide a sustained increase in maternal and fetal serum. To minimize 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 pre-specified 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 (4400g, 15 minutes). 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 off. Within 30 minutes of the sample collection, specimens will be transferred to a laboratory. The samples will be centrifuged (1000g, 15 minutes) 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 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 hours 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- and antioxidant enzymes, melatonin synthesizing 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 wellbeing, mortality and morbidity (Tables 1-4).

Table 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

Table 2: Maternal morbidity.

Markers of maternal morbidity: at recruitment and at least weekly until delivery.
Blood pressure
Complete blood count
Liver function tests (AST, ALT, GTT, bilirubin)
Renal function tests (urea, creatinine, uric acid)

Table 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 sulphate and corticosteroids.
Use of (multi) vitamins and dietary supplements

Table 4: Neonatal outcomes.

Neonatal outcomes:
Sex
Apgar scores (1, 5, 10 minutes)
Birth weight
Cord artery 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, necrotizing enterocolitis, abnormal neurology,

mortality prior to discharge

Follow up of women and infants: Participants will be followed weekly for the duration of the trial. The routine clinical care of participants, including ultrasound scans, will not be affected by participation in the study. During the weekly follow-up, fetal and maternal wellbeing 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, 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 three days post-partum, 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 scans, 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 randomized-controlled trial. This trial will provide data for the establishment of the use of maternal melatonin treatment in fetal growth restriction.

The results obtained in this trial will be compared to historic cohorts of untreated fetal growth restriction 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, 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 analysis of variance, including the baseline value as a covariate. Levels of oxidative stress in the maternal blood sample obtained immediately prior to birth will be compared to samples derived from the historic cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will be compared to data obtained from the previously described historic cohorts. Clinical data regarding maternal wellbeing, pregnancy outcome and neonatal outcome will be compared to 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 hours.

Trial discontinuation or modification

The participants can 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 are within the natural history of fetal growth restriction and are not part of the discontinuation criteria.

Ethics and dissemination: This study has been approved by the Monash Health Human Research Ethics Committee B (HREC 12133B). Data will be presented at relevant conferences and published in peer-reviewed journals.

DISCUSSION

The use of melatonin as an antenatal antioxidant therapy in fetal growth restriction 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 randomized-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 study to study the clinical and biochemical benefit of melatonin treatment in preeclampsia has been initiated.

AUTHORS' CONTRIBUTIONS

NOA: research, contribution of original material, editing and approval of final manuscript. SLM, GJ, EMW: editing and approval of final manuscript.

FUNDING STATEMENT

This work was supported by the 5Point Foundation.

COMPETING INTERESTS

NOA is funded by a Monash Graduate Scholarship and a Monash International Postgraduate Research Scholarship.

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