PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction – a phase I pilot clinical trial: study protocol
AUTHORS	Alers, Nicole; Jenkin, Graham; Miller, Suzanne; Wallace, Euan

VERSION 1 - REVIEW

REVIEWER	Vittorio Unfer A.G. Un. Co. Obstetrics and gynaecology center
REVIEW RETURNED	10-Oct-2013

GENERAL COMMENTS	1) I suggest to have as main outcome a clinical one 2) Power analysis should be performed using the historic cohort and based on this, evaluate the number of patients that need to be recruited 3) Please explain the rational of the dosage 8mg/day 4) melatonin absorption varies between subject, therefore, data analysis should be performed having this in mind.

REVIEWER	Alessandro Ghidini Inova Alexandria Hospital USA
REVIEW RETURNED	01-Nov-2013

GENERAL COMMENTS	This study protocol of a phase I pilot trial on 12 women aimed at exploring the ability of melatonin to mitigate the morbidity of preterm (<34 weeks) fetal growth restriction (EFW <10th centile). Comments: - Will information be collected on intake of other antioxidants, eg in the diet (dark chocolate, vitamin E, vitamin C, pomegranate, etc)? it is possible that once women realize the purpose of the trial, they may check online for other antioxidants and increase their dietary intake. - Since one of the goals is to determine “the level … of oxidative stress in the placenta”, do the authors plan to collect the placentas and examine them? - In the Introduction the authors do not mention the numerous trials on other antioxidants (eg vitamins C and E) for conditions associated with placenta oxidative stress (e.g. severe and preterm preeclampsia). Why would melatonin be different? - Page 8 line 16: after “severe fetal asphyxia” add “in animal models” - Methods: what is the definition of “on abnormal fetoplacental Doppler study of the uterine artery, umbilical artery, MCA or ductus

	venosus"? would notching in 1 uterine artery qualify? Or would they use pulsatility index? In the latter case, what threshold (90th centile? 95th centile?) - How did the authors come up with the dosage of 4 mg BID as optimal dose to test? Since melatonin is safe at higher dosages, why not start with a higher dose? Otherwise negative findings may be ascribed to inadequate dosing. - Table 2: I recommend adding uric acid to the serum markers monitored, as it is involved in the antioxidant system - Table 5: I suggest adding umbilical artery pH to the neonatal outcome measures

REVIEWER	Cathy Vaillancourt INRS-Institut Armand Frappier Laval, QC, Canada
REVIEW RETURNED	03-Nov-2013

GENERAL COMMENTS	This is a well-design clinical trial study, melatonin as a real preventive and or therapeutic potential in obstetric complication associated with increases oxidative stress (such as preeclampsia, pre-term birth, fetal growth restriction and diabetes gestational). However, I was some comments and suggestions. As showed in 2008 by Lanoix et al. (J Pineal Res), the human placenta produces melatonin de novo and expresses its receptors. It will be important to verify if melatonin treatment affect placental production of melatonin (arylalkylamine N-acetyltransferase (AANAT) activity/expression) and/or melatonin receptors expression. It has been shown both "in vitro" and "in vivo" studies that there is possibly an inhibition of the synthesis of melatonin by the pineal gland, and a production of this indolamine by immune cells (Markus, Cecon et al. 2013). This could also be the case in placenta where maternal pineal melatonin (or administration of melatonin) could affect placental production or vice-versa. Lanoix et al. has also demonstrated that melatonin (Mol Cell Endocrinol. 2013) in primary villous trophoblast (in vitro) prevents the hypoxia-reoxygenation-induced oxidative stress and mitochondrial apoptosis. They also showed that melatonin increases antioxidant enzymes activity and expression. This study should be cited. Also, it will be of interest to look at the placental antioxidant enzymes expression and activity. The authors have recently published a study protocol, in which they administrated melatonin to PAMPR women (BMJ Open 2013). What is the relationship between the two studies? Will the authors analyze the data from the two studies together to compare them and increase their cohort? It will be interesting to do it. How placenta samples were collected? This is an important step that could affect gene expression, etc. Will you take more than one placental biopsies by placenta? Placenta is a heterogeneous tissue thus, to reduce the bias related to the physiological difference in gene expression within the same placenta depending on the sampling site (Pidoux, et al. 2004; Sood, et al. 2006; Wyatt, et al. 2005), multistage unbiased random sampling should be applied in order to give all parts the same chance of being chosen (Mayhew 2008; Wyatt et al. 2005). To ensure that placental analysis are

	representative of the placenta as a whole 4-5 tissues samples should be collected (and snap frozen in liquid nitrogen) from each placenta using a stratified random sampling method and the 4-5 tissue samples pooled for the RNA extraction.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1
(Vittorio Unfer, A.G. Un. Co.Obstetrics and gynaecology center)
- I suggest to have as main outcome a clinical one
We agree that the clinical outcomes will, ultimately, be the main issue. However, as detailed above, we wished to first demonstrate proof-of-principle than maternal administration of melatonin could exert an anti-oxidant effect in the fetus and placenta. This information will be used to inform the design of an appropriate RCT in the future which will have a clinical outcome, eg neurodevelopment, as the primary outcome.

- Power analysis should be performed using the historic cohort and based on this, evaluate the number of patients that need to be recruited
Unfortunately, we have no historic data addressing the possible effects of melatonin. This study will be the first study to show an effect, if any, and will be the basis of sample size calculations of future studies.

- Please explain the rational of the dosage 8mg/day
We have now provided the rational for this.

- Melatonin absorption varies between subject, therefore, data analysis should be performed having this in mind.
Thank you. We will measure levels of melatonin in the maternal and fetal circulation, and placenta. We will be able to relate the level of melatonin to the time of intake of melatonin. We will analyse levels of oxidative stress compared to the serum levels of melatonin.

Reviewer 2
(Alessandro Ghidini, Institution and Country Inova Alexandria Hospital USA)

This study protocol of a phase I pilot trial on 12 women aimed at exploring the ability of melatonin to mitigate the morbidity of preterm (<34 weeks) fetal growth restriction (EFW <10th centile).
Comments:
- Will information be collected on intake of other antioxidants, eg in the diet (dark chocolate, vitamin E, vitamin C, pomegranate, etc)? it is possible that once women realize the purpose of the trial, they may check online for other antioxidants and increase their dietary intake.
Thank you. We had not initially considered collecting this information but we will now, assessing dietary and supplemental intake of other sources of anti-oxidants.

- Since one of the goals is to determine “the level … of oxidative stress in the placenta”, do the authors plan to collect the placentas and examine them?
We do. We have amended the manuscript to describe more clearly how we plan to collect and examine the placentae.

-In the Introduction the authors do not mention the numerous trials on other antioxidants (eg vitamins C and E) for conditions associated with placenta oxidative stress (e.g. severe and preterm preeclampsia). Why would melatonin be different?
Melatonin has been shown to be significantly more effective than Vit C and E in protecting cells from

oxidative stress (Martin et al 2000, Reiter et al 2009, Milczarek et al 2010, Lowes et al 2013). Additionally, the biosafety profile of vitamins C and E is suboptimal. Prolonged pharmacological use of vitamin E compromises the immune system of neonates (Aversa 2012), and lead to an increase of sepsis and necrotizing enterocolitis in neonates (Johnson 1985, Brion 2003). In the absence of oxidative stress, vitamin E can actually induce apoptosis (Then 2009), whilst vitamin C can act as a pro-oxidant (Podmore 1998). In pregnant women vitamin C and E therapy increases the risk of gestational hypertension and premature rupture of membranes (Conde 2011). In contrast, melatonin has a remarkably benign biosafety profile. Long term treatment with high doses of melatonin, have been used in adults and children without any problems. Melatonin has also been administered in high doses (both orally and IV) in compromised neonates, without any toxicity. Animal studies studying the developmental toxicity of melatonin have not been able to identify any serious toxicity. Because of this remarkably good biosafety profile, we believe that melatonin as an antioxidant treatment is a worthy candidate in the management of fetal growth restriction. Furthermore, unlike vit C and E, melatonin induces endogenous anti-oxidant enzymes.

- Page 8 line 16: after “severe fetal asphyxia” add “in animal models”
Now added.

- Methods: what is the definition of “on abnormal fetoplacental Doppler study of the uterine artery, umbilical artery, MCA or ductus venosus”? would notching in 1 uterine artery qualify? Or would they use pulsatility index? In the latter case, what threshold (90th centile? 95th centile?)
We have amended the methods section to provide more detailed inclusion criteria regarding fetoplacental Doppler studies.

- How did the authors come up with the dosage of 4 mg BID as optimal dose to test? Since melatonin is safe at higher dosages, why not start with a higher dose? Otherwise negative findings may be ascribed to inadequate dosing.
We have added further details about our chosen dosage regimen. To our knowledge, the highest, long term dose of melatonin during pregnancy that has been used is 3mg once daily. We are not aware of any published human studies in which melatonin has been used in pregnancies with fetal growth restriction. We have chosen for a dose of 4mg BD, as we envisage that 3mg once daily will be insufficient to result in an antioxidant effect. This dose is based on our own experimental research, with other human melatonin studies in mind (during healthy pregnancies and in neonates). We agree that this dose may be conservative and we appreciate that we run the risk of obtaining a negative result due to inadequate doses. However, even at this dosage maternal melatonin levels increase about 40-fold.

- Table 2: I recommend adding uric acid to the serum markers monitored, as it is involved in the antioxidant system
Thank you. Uric acid was one of our serum markers. We have amended the table to reflect this.

- Table 5: I suggest adding umbilical artery pH to the neonatal outcome measures
Thank you. At our institution, cord lactate levels are measured as routine. A cord pH is more difficult to acquire due to limited access to pH analyzer. However, we will explore this further. It is a sensible recommendation.

Reviewer 3
(Cathy Vaillancourt, INRS-Institut Armand Frappier Laval, QC, Canada)

This is a well-design clinical trial study, melatonin as a real preventive and or therapeutic potential in obstetric complication associated with increases oxidative stress (such as preeclampsia, pre-term

birth, fetal growth restriction and diabetes gestational). However, I was some comments and suggestions.

- As showed in 2008 by Lanoix et al. (J Pineal Res), the human placenta produces melatonin de novo and expresses its receptors. It will be important to verify if melatonin treatment affect placental production of melatonin (arylalkylamine N-acetyltransferase (AANAT) activity/expression) and/or melatonin receptors expression. It has been shown both “in vitro” and “in vivo” studies that there is possibly an inhibition of the synthesis of melatonin by the pineal gland, and a production of this indolamine by immune cells (Markus, Cecon et al. 2013). This could also be the case in placenta where maternal pineal melatonin (or administration of melatonin) could affect placental production or vice-versa.
Thank you. We had planned to measure melatonin in the placenta and to measure the gene expression of both the two synthesizing enzymes (AANAT, HIOMT) and the two melatonin receptors (MT1 and MT2).

- Lanoix et al. has also demonstrated that melatonin (Mol Cell Endocrinol. 2013) in primary villous trophoblast (in vitro) prevents the hypoxia-reoxygenation-induced oxidative stress and mitochondrial apoptosis. They also showed that melatonin increases antioxidant enzymes activity and expression. This study should be cited. Also, it will be of interest to look at the placental antioxidant enzymes expression and activity.
We have now cited this paper. We plan to measure antioxidant enzyme expression. We have amended the manuscript to reflect this.

The authors have recently published a study protocol, in which they administrated melatonin to PAMPR women (BMJ Open 2013). What is the relationship between the two studies? Will the authors analyze the data from the two studies together to compare them and increase their cohort? It will be interesting to do it.
The two studies are related but distinct. The PAMPR trial will recruit women with preeclampsia (with or without IUGR) whereas this trial will only recruit women with IUGR. There is a different dosage regimen in the PAMPR trial and the likely duration of treatment will be much shorter, commencing at more advanced gestations. That said, a number of outcome variables are shared and it will be possible for us to compare and contrast these outcomes. This will hopefully provide us with some insights into likely optimum doses for a future RCT.

- How placenta samples were collected? This is an important step that could affect gene expression, etc. Will you take more than one placental biopsies by placenta? Placenta is a heterogeneous tissue thus, to reduce the bias related to the physiological difference in gene expression within the same placenta depending on the sampling site (Pidoux, et al. 2004; Sood, et al. 2006; Wyatt, et al. 2005), multistage unbiased random sampling should be applied in order to give all parts the same chance of being chosen (Mayhew 2008; Wyatt et al. 2005). To ensure that placental analysis are representative of the placenta as a whole 4-5 tissues samples should be collected (and snap frozen in liquid nitrogen) from each placenta using a stratified random sampling method and the 4-5 tissue samples pooled for the RNA extraction.
We have amended the paper to describe the collection and processing of placental specimens in more detail. To standardize the site of biopsy, we take 6 full thickness placental samples from the same region in the placenta. The amnion and chorion are stripped off and the samples are pooled for RNA extraction. We have recently become aware of the Mayhew 2007 paper and the flaws of different methods of sampling, but had already started the collection of placental samples in our historic cohort by standardizing the site of placental biopsy. We will continue to do so in this trial so that the comparison between the different cohorts is not biased. We realize that by taking samples

from one standardized site, we cannot draw any definite conclusions about the placenta as a whole organ. In the planned future RCT we will use the stratified random sampling method.