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A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

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Abstract

Introduction

High levels of oxidative stress can have considerable impact on the outcomes of in-vitro fertilization (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant anti-oxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3-4mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose-response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Methods and analyses

We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2mg twice per day (n=40); melatonin 4mg twice per day (n=40) and melatonin 8mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilization rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy- 2'-deoxyguanosine (8-OHdg) at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores, and pregnancy complications, comparing outcomes between groups. This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.

Ethics and dissemination:

Ethical approval has been obtained from Monash Health HREC (Ref: 13402B), Monash University HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals.

Registration Details: Version 1. Australian New Zealand Clinical Trials Registry, ACTRN: ACTRN12613001317785. The complete WHO Registration Data Set, as described by the SPIRIT 2013 guidelines is available for viewing at <https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=365376>

Strengths and limitations

- This trial is a well-designed pilot study to achieve biochemically and clinically relevant outcomes.
- Significant preliminary research has been performed in order to appropriately design the study to fill gaps in current knowledge.
- This is the first placebo controlled dose-finding trial of melatonin in the field of infertility world-wide and if successful, has the potential to provide a foundation for future large randomised controlled trials.
- Because of constraints of such a pilot study in the infertile population, relatively small numbers of participants are anticipated. To help minimise the effect of this, a cross-over arm will be introduced. It is expected that this limitation will be overcome in future trials that will be designed based on this study.

Introduction

Humans are aerobic organisms and when oxygen is utilised in metabolic processes, free oxygen radicals are created as a consequence. These radicals are oxidants and contain 'free' valence electrons, making them highly unstable and therefore reactive, capable of causing injury to cells. This occurs by accepting electrons from another molecule (a reductant) in order to reach stability. In doing so, the chemical structure of the reductant is changed, and may no longer be able to perform its designated function. In fact, often the reductant can then produce or sometimes even become an oxidant having the potential to cause oxidative stress in its own right.¹ Anti-oxidative agents are present endogenously and can also be administered exogenously. They are designed to reduce free radicals by donating electrons to stabilise the free radical.² The term 'reactive oxygen species' (ROS), not only includes free radicals but also molecularly stable non-radical molecules which contain oxygen and are capable of causing oxidative stress, such as hydrogen peroxide (H₂O₂).³ While ROS are necessary for essential physiological processes, an overabundance can result in cellular and tissue damage and this is commonly referred to as 'oxidative stress'.⁴ Oxidative stress can be measured in bodily fluids by markers including 8-hydroxy- 2'-deoxyguanosine (8-OHdg).⁵

Over recent years, the potential role of oxidative stress in the outcomes of assisted reproductive technology (ART) has been gaining increasing attention, in particular with regards to in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). This is perhaps not surprising given that ART exposes both oocytes and embryos to high levels of superoxide free radicals during gamete and embryo culture⁶. Indeed, in addition to the culture conditions, *in vitro* the oocytes are not afforded the normal protection of the antioxidant-rich follicular fluid or surrounding cumulus cells, leaving them more susceptible to oxidative damage.^{7,8} Reproductive processes such as chromosome segregation (resulting in euploid oocytes), polar body extrusion (necessary for haploid oocytes), fertilization and early cleavage (to avoid arrested embryos) require energy. As the average age of women seeking fertility treatment rises, the level of ROS and subsequent oxidative stress increases, resulting in mitochondrial DNA mutations which in turn affect ATP production in the oocyte. Without the necessary ATP, required cellular processes cannot occur correctly, having a direct effect on the quality of the oocyte, embryo and the final outcome of the IVF procedure.^{9,10}

It has been shown that oocyte quality begins to deteriorate immediately following ovulation. This has been thought to be an inflammatory driven oxidative stress process¹¹ whereby the production of cytokines and proteases is associated with an increase in ROS which in turn impair oocyte maturation.¹²⁻¹⁴ Evidence of oxidative damage in *in vitro* oocytes has been shown to exist as early as 8 hours after ovulation.¹⁵ Nor surprisingly it has been shown that high levels of the oxidative stress in the follicular fluid of infertile women are associated with poor oocyte maturation and embryo quality, and that inducers of oxidative stress can be used to inhibit oocyte maturation.^{5,12}

1 Together, these observations highlighting the importance of oxidative stress in the developing
2 follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.
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5 Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has
6 important oxygen-scavenging properties which naturally mitigate oxidative stress by both
7 neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.¹⁶⁻¹⁸
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9 Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many
10 medical conditions in which oxidative stress has been implicated including diabetes, glaucoma,
11 irritable bowel syndrome and fertility.¹⁹⁻²²
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14 The effects of melatonin supplementation on culture media,^{12, 21, 23-27} gametes,^{5, 15, 28} embryos^{15,}
15 ^{29, 30} and luteal function³¹⁻³⁵ have been identified as useful areas for investigation. Human clinical
16 studies have begun recently, with emphasis on oral supplementation of melatonin during the
17 stimulation cycle and its effects on oocyte and embryo quality.^{12, 36-39}
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20 Importantly, melatonin has a remarkably benign safety profile in both animal and human studies.
21 A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses
22 at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival,
23 however, also resulted in a reduction in healthy follicles. They concluded that doses of greater
24 than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.⁴⁰ The maternal
25 lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was
26 observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans.
27 Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have
28 been reported for up to doses of 200mg/kg/day.⁴¹ There have been several randomised controlled
29 trials addressing high doses of melatonin in human adults and children,⁴²⁻⁴⁶ and reports have
30 established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.⁴⁷
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39 Rationale

40 In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5
41 cycles resulting in live birth⁴⁸. That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there
42 is much room for further improvement, if only to meet societal expectations and reduce
43 healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF
44 outcomes is therefore merited. Several human and animal studies support the use of melatonin in
45 the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant
46 properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and
47 embryos,⁴⁹ with intrafollicular melatonin concentrations being significantly higher than those
48 found in serum, again suggesting a physiological role in reproduction.^{39, 67, 68} The human studies
49 addressing the use of melatonin in infertility treatment undertaken to date have been small and
50 not placebo-controlled. None have attempted to identify an optimal dose.^{36-38, 50} Further,
51 interpretation of any outcomes have been hampered by the within-patient comparison design,
52 where patients act as their own controls.^{12, 39, 51}
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1 We have designed this study to be the first placebo-controlled, dose-finding clinical trial to
2 investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI
3 treatment.
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8 **Aims**

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10 The overall aim of this trial is to determine whether oral melatonin administration can improve the
11 outcomes of IVF/ICSI. In order to address this, we will determine whether melatonin
12 administration has a dose-response effect in women undergoing IVF/ICSI on:
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- 15 1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-
16 deoxyguanosine (8-OHdg), progesterone, oestradiol)
 - 17 2. sonographic markers of follicle health
 - 18 3. patient sleepiness
 - 19 4. pregnancy rate following IVF/ICSI
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28 **Methods and analyses**

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30 This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.⁵² Recruitment will
31 commence in July 2014. This will occur over two years. Analysis and dissemination will occur after
32 this period of time. The study is expected to be completed by February 2017.
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37 **Study design**

38 Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial
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43 **Subjects**

44 We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.
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50 **Study setting**

51 Patients will be recruited from Monash IVF infertility clinics in Melbourne, Australia over a period
52 of 2 years.
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Interventions to be measured

This trial will have four arms. All capsules will be indistinguishable from each other.

1. Placebo capsule taken twice per day
2. 2mg melatonin capsule twice per day (4mg/d total)
3. 4mg melatonin capsule twice per day (8mg/d total)
4. 8mg melatonin capsule twice per day (16mg/d total)

Primary Outcome

Clinical pregnancy rate, defined as the presence of a live pregnancy in the uterine cavity at a transvaginal ultrasound(TVUS) at 6-8 weeks' gestation

Secondary outcomes

See *Table 1* for details.

Sample Size

As this is a pilot dose-finding study, without precedence on which to base accurate power calculations, a power calculation has not been performed. Part of our aim is to provide clarification allowing for larger randomised trials to be designed in the future. We have chosen a convenience sample of 160 participants, 40 in each of the four groups, in order to assess plausible morphological, biochemical and sonographic surrogate markers for oocyte quality, as well as to provide an indication of the effect size for each dose for our primary outcome, clinical pregnancy rate. Conservatively estimating that, in total, 50% of patients (n=80) do not get pregnant after their first cycle, we may assume that 60 patients will participate in the crossover arm (allowing for drop-outs) in their second cycle and be allocated to a different treatment group.

Inclusion criteria

1. Undergoing first cycle of IVF or ICSI
2. Age between 18 and 45
3. Body mass index (BMI) between 18 and 35

4. Undergoing a GnRH antagonist cycle (without OCP scheduling)

Exclusion criteria

1. Current untreated pelvic pathology – moderate to severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease, uterine malformations, Asherman's syndrome and hydrosalpinx.
2. Currently enrolled in another interventional clinical trial
3. Concurrent use of other adjuvant therapies (eg. Chinese herbs, acupuncture)
4. Current pregnancy
5. Malignancy or other contraindication to IVF
6. Autoimmune disorders
7. Undergoing preimplantation genetic diagnosis (PGD)
8. Hypersensitivity to melatonin or its metabolites
9. Concurrent use of any of the following medications
 - a. Fluvoxamine (eg. Luvox, Movox, Voxam)
 - b. Cimetidine (eg. Magicul, Tagamet)
 - c. Quinolones and other CYP1A2 inhibitors (Ciprofloxacin, Avalox)
 - d. Carbamazepine (eg. Tegretol), rifampicin (eg. Rifadin) and other CYP1A2 inducers
 - e. Zolpidem (eg. Stilnox), zopiclone (eg. Imovane) and other non-benzodiazepine hypnotics
10. Inability to comply with trial protocol

Concomitant care

Aside from trial protocol and inclusion/exclusion criteria listed above, standard concomitant care, such as folic acid supplements, is permitted.

Participant enrolment

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This trial will begin recruitment at the level of the infertility specialist. Once identified as requiring IVF/ICSI, and satisfying inclusion and exclusion criteria, the patient will be provided with detailed written information about the trial protocol. After approximately one week, the patient will be approached and written informed consent will be obtained by the principle investigator at the first visit with the infertility clinic nurse. Baseline bloods will then be taken and the patient will be randomised by the principle investigator (see below) to one of the study arms and provided with the trial medication. The participant will commence taking the trial medication on the day that ovarian stimulation injections begin (Day 2-3 of menses) at 0800 and 2200 each day, with the last capsule being taken at 2200 the night before oocyte collection.

For a summarised participant timeline, See *Figure 1* and *Figure 2*.

Allocation concealment, blinding and randomisation

Each treatment arm will be randomly allocated a letter (A, B, C or D) by way of opaque sealed envelope. Randomisation will be computerised and patients will be randomised at a ratio of 1:1:1:1 to one of the groups, A-D, using the minimisation method, a method of randomisation accounting for factors known to affect the outcome used in small trials to prevent selection bias.⁵³

With a crossover design improved patient recruitment is very likely because all non-pregnant participants will be guaranteed at least one cycle with one of the doses of melatonin.⁵⁴ After completing the first study cycle, those participants that are not pregnant will be recruited for the cross-over cycle in which they will be assigned the next treatment arm. For example, if a participant were allocated to treatment group A in their first cycle and did not become pregnant, they would be offered allocation to group B in their second cycle and so on.

Participants will be blinded by receiving identical-appearing unmarked capsules. All trial researchers will be blinded to treatment allocation group until after analyses are performed at the completion of the trial. A Data Safety Monitoring Committee (see *Adverse events* for details) and dispensing pharmacy will be aware of allocation to allow identification of any unbalanced or multiple serious adverse events that may necessitate emergency unblinding of researchers and participants.

Adherence and retention

In order to ensure the integrity of study data, participant adherence to trial protocol will be assessed on a medication administration record updated daily by the participant. At oocyte collection, participants will return medication bottles and compliance will be confirmed by counting remaining tablets. This will be recorded on individual patient compliance forms and patient record forms. Every reasonable effort will be made from the time of enrolment until the end of follow-up to maintain contact with and maintain subject participation in the trial.

Analysis plan

SPSS v22.0 (IBM, Armonk, New York) will be used for data analysis. Primary analysis will occur by the intention-to-treat principle. A secondary analysis will be performed for 'actual treatment received'. Statistical analyses will be performed using Chi-square tests for dichotomous categorical outcome variables. Clinical and demographic data will be analysed with parametric tests if they are normally distributed (ANOVA with Bonferroni adjustment), otherwise appropriate non-parametric tests will be implemented (Kruskal-Wallis for between group analyses). Within patient changes (paired data), for example when comparing serum levels of melatonin at baseline and after treatment, will be compared using repeated measures ANOVA or Wilcoxon signed rank test depending on normality of data. $P < 0.05$ will be considered statistically significant. Where statistically sound, adjusted odds ratios will be calculated to account for confounders or effect modifiers. To account for missing data, two analyses will be run, with the first excluding missing values and the second by imputation of missing values to determine how conclusions are affected.

Adverse events and data safety and monitoring

An independent Data Safety and Monitoring Committee (DSMC) has been established at Monash Health in order to monitor occurrences of adverse events. The principle investigator will be available by telephone at all times during the trial and participants will be provided with contact details in case of any adverse events. Participants will also be interviewed at the time of their ultrasound and the day of oocyte collection to ascertain the occurrence of any adverse events. Serious adverse events will be recorded separately and followed up until resolution. Such events will be reported to the Monash Health, Monash University, Monash Surgical Private Hospital and Epworth HealthCare Human Research Ethics Committees, the Monash Health DSMC, the Monash Health Therapeutics Committee and, if directed by HREC, to the Australian Therapeutics and Goods Administration.

Trial modification and discontinuation

In the absence of adverse events, the medication regimen will not be modified once commenced. If other protocol changes are deemed necessary by the investigating team, ethics approval will be sought from all approving Human Research Ethics Committees. Once approved, protocol amendments will be notified to all investigators, administrators (eg. dispensing pharmacy) and trial participants via telephone.

Patients are permitted to discontinue their inclusion in the trial at any point in time at their request or following an unexpected serious adverse event as described above. The DSMC will perform an interim analysis specifically assessing adverse events after 50% of participants have

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been recruited. Otherwise, the trial will cease follow-up once patients have given birth from a trial treatment cycle.

Data collection, informed consent forms and confidentiality

Data will be recorded in both hardcopy and electronic form. Hardcopies will be stored in a secured filing cabinet at the administering institution. Electronic copies will be stored in encrypted files on a password protected computer. All data will be kept for 15 years, following this time, hardcopies will be destroyed by shredding or burning and electronic copies will be deleted by formatting. Organic samples will be stored until analysis in a secured locked temperature-controlled freezer at the Monash Institute of Medical Research that can only be accessed by authorized staff. Samples and participant records will not contain any directly identifiable information. No additional biological samples will be kept for use in ancillary studies.

For access to data collection forms, including sleep diaries, medication compliance and informed consent forms please contact the principle investigator.

Ethics and dissemination

Ethical approval has been obtained from Monash Health HREC (Ref:13402B), Monash University HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals. De-identified summary results will also be made publically available on the Monash IVF website.

Discussion

The MIART trial has the potential to improve IVF treatment protocols, with both immediate and translational benefit to patients. If melatonin is found to improve outcomes after IVF/ICSI, it may become a routine part of management of the infertile couple. We aim to set precedence and a framework by which others may structure further investigation into melatonin and other adjuvant therapies in the future. The intention of this study is as a pilot trial, primarily designed to provide un-biased data as a foundation for further research into this area. It is anticipated that the effect size observed in this trial will be useful to more appropriately power subsequent RCTs with the most effective dose of melatonin. In summary, MIART will be the first trial designed to determine a dose-response relationship of melatonin on biochemical and physiological markers of follicle health and also clinical pregnancy rates.

Contributors:

The idea for the trial was conceived by SF, LR, and EW. SF was involved in research design and primary writing of study protocol and manuscript. TO was involved in the design and writing of the manuscript. LR and EW were involved in the trial design, writing, editing and approval of the final manuscript.

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Conflict of interest statement

SF is supported by an NHMRC Postgraduate Scholarship. No conflicts of interest are identified for SF, EW or TO. BV and LR are minority shareholders in Monash IVF. LR has received unconditional research and educational grants from MSD® and Merck Serono®. The trial sponsor had no influence on trial design, implementation, analysis or presentation.

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Figure Legends

Table 1: Clinical Secondary Outcomes

OHSS: Ovarian Hyperstimulation syndrome; hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

^aReyner L, Horne J. Falling asleep whilst driving: are drivers aware of prior sleepiness? Int J Legal Med 1998;111:120-3

Figure 1: Participant timeline schematic - first cycle

^aPlacebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin

Table 1: Clinical Secondary Outcomes

Clinical outcomes	Definition
Live birth rate	Birth of a live baby after 24 weeks gestation
Miscarriage rate	Loss of a diagnosed clinical pregnancy before 20 weeks gestation
Sleepiness score	Based on Karolinska ^a sleepiness scale during trial medication administration
Pregnancy complication rate	Including OHSS, multiple pregnancy, congenital or chromosomal abnormalities, stillbirth, preeclampsia, delivery before 34 weeks, delivery between 34 and 37 weeks, placenta praevia, gestational diabetes, low birthweight
Embryological outcomes	
Total number of oocytes collected	
Oocyte maturity	
Total number of embryos	
Embryo quality	
Fertilization rate	The proportion of oocytes that become fertilised
Utilisation rate	Proportion of zygotes undergoing embryo transfer or cryopreservation to oocytes fertilised
Biochemical outcomes	
Biochemical pregnancy rate	Presence of serum hCG level of >25IU/l on Day 16 after embryo transfer
Melatonin levels in serum	At baseline and prior to oocyte collection
Melatonin levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
8-OHdg levels in serum	At baseline and prior to oocyte collection
8-OHdg levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
Oestradiol and Progesterone levels in serum	Taken at baseline, during treatment and at the time of oocyte collection
Sonographic outcomes	
Follicular blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection
Uterine artery blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection

OHSS: Ovarian Hyperstimulation syndrome; hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy-2'-deoxyguanosine

^aReyner L, Horne J. Falling asleep whilst driving: are drivers aware of prior sleepiness? *Int J Legal Med* 1998;111:120-3

Figure 1: Participant timeline schematic - first cycle

	Study Period							
	Enrolment	Trial medication beings	Post allocation			Close out		
Timepoint (days)	-7	0	8-10	14	19	68	299	
ENROLMENT								
Eligibility Screen	x							
Informed Consent	x							
Allocation	x							
INTERVENTIONS								
Trial Medication ^a		x	—————	x				
ASSESSMENTS								
Baseline blood tests	x							
Blood tests after treatment				x				
Follicular fluid melatonin and 8-OHdg				x				
Measurement of follicular and uterine blood flow			x					
Oocyte assessments				x				
Embryo assessments				x	—————	x		
Sleepiness scores		x	—————	x				
Pregnancy complication rates and outcomes						x	—————	x
Followup hCG					x	—————	x	

^aPlacebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

	Study Period							
	Confirmation of pregnancy failure	Second trial medication begins*	Post reallocation			Close out		
Timepoint (days)		0	8-10	14	19	68	299	
INTERVENTIONS								
Offer reallocation	x							
Trial Medication ^a		x	—————	x				
ASSESSMENTS								
Oocyte assessments				x				
Embryo assessments				x	—————	x		
Sleepiness scores		x	—————	x				
Pregnancy complication rates and outcomes						x	—————	x
Followup hCG					x	—————	x	

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin



STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Page(s)	Section/item	ItemNo	Description
Administrative information			
1	Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym
3	Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry
3		2b	All items from the World Health Organization Trial Registration Data Set
3	Protocol version	3	Date and version identifier
13	Funding	4	Sources and types of financial, material, and other support
1,2,13	Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors
2		5b	Name and contact information for the trial sponsor
13		5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities
11		5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)
Introduction			
5-7	Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention
5-7		6b	Explanation for choice of comparators
7	Objectives	7	Specific objectives or hypotheses
7-11	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)
Methods: Participants, interventions, and outcomes			
7	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained

1	8-9	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)
2				
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4	7-8	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
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8	11		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)
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12	10		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
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16	9		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
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19	8, Table	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended
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26	Fig 1 and	Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)
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31	8	Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations
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35	9-10	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
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43	10	Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions
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50	10	Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
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54	10	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
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58	10	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
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	10		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

Methods: Assignment of interventions (for controlled trials)

Allocation:

Methods: Data collection, management, and analysis

1				
2	12	Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
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11	10		18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
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15	12	Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
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21	11	Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol
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26	11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
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28	11		20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)
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Methods: Monitoring

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34	11	Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed
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41	11		21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial
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46	11	Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct
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51	11	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
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Ethics and dissemination

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56	12	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
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59	11	Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
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1	10	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
2				
3	N/A		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
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6	12	Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial
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11	13	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site
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14	12	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators
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18	11	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
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21	12	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions
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27	12		31b	Authorship eligibility guidelines and any intended use of professional writers
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29	12		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code
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33		Appendices		
34	Available	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates
35	on			
36	request			
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39	12	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable
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*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.

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BMJ Open

A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

Journal:	<i>BMJ Open</i>
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Secondary Subject Heading:	Reproductive medicine
Keywords:	Reproductive medicine < GYNAECOLOGY, Subfertility < GYNAECOLOGY, Sex steroids & HRT < DIABETES & ENDOCRINOLOGY

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Manuscripts

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4 **A pilot double-blind randomised placebo-controlled dose-response trial assessing**
5 **the effects of melatonin on infertility treatment (MIART): study protocol**
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30 **Running Title:** Melatonin in ART - MIART randomised trial: study protocol

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33 **Keywords:**

34 Melatonin, assisted reproductive technology (ART), in-vitro fertilization, oxidative stress, protocol

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Abstract

Introduction

High levels of oxidative stress can have considerable impact on the outcomes of in-vitro fertilization (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant anti-oxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3-4mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose-response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Methods and analyses

We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2mg twice per day (n=40); melatonin 4mg twice per day (n=40) and melatonin 8mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilization rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy- 2'-deoxyguanosine (8-OHdg) at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores, and pregnancy complications, comparing outcomes between groups. This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.

Ethics and dissemination:

Ethical approval has been obtained from Monash Health HREC (Ref: 13402B), Monash University HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals.

Registration Details: Version 1. Australian New Zealand Clinical Trials Registry, ACTRN: ACTRN12613001317785. The complete WHO Registration Data Set, as described by the SPIRIT 2013 guidelines is available for viewing at <https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=365376>

Strengths and limitations

- This trial is a well-designed pilot study to achieve biochemically and clinically relevant outcomes.
- Significant preliminary research has been performed in order to appropriately design the study to fill gaps in current knowledge.
- This is the first placebo controlled dose-finding trial of melatonin in the field of infertility world-wide and if successful, has the potential to provide a foundation for future large randomised controlled trials.
- Because of constraints of such a pilot study in the infertile population, relatively small numbers of participants are anticipated. To help minimise the effect of this, a cross-over arm will be introduced. It is expected that this limitation will be overcome in future trials that will be designed based on this study.

Introduction

Humans are aerobic organisms and when oxygen is utilised in metabolic processes, free oxygen radicals are created as a consequence. These radicals are oxidants and contain 'free' valence electrons, making them highly unstable and therefore reactive, capable of causing injury to cells. This occurs by accepting electrons from another molecule (a reductant) in order to reach stability. In doing so, the chemical structure of the reductant is changed, and may no longer be able to perform its designated function. In fact, often the reductant can then produce or sometimes even become an oxidant having the potential to cause oxidative stress in its own right.¹ Anti-oxidative agents are present endogenously and can also be administered exogenously. They are designed to reduce free radicals by donating electrons to stabilise the free radical.² The term 'reactive oxygen species' (ROS), not only includes free radicals but also molecularly stable non-radical molecules which contain oxygen and are capable of causing oxidative stress, such as hydrogen peroxide (H₂O₂).³ While ROS are necessary for essential physiological processes, an overabundance can result in cellular and tissue damage and this is commonly referred to as 'oxidative stress'.⁴ Oxidative stress can be measured in bodily fluids by markers including 8-hydroxy- 2'-deoxyguanosine (8-Ohdg).⁵

Over recent years, the potential role of oxidative stress in the outcomes of assisted reproductive technology (ART) has been gaining increasing attention, in particular with regards to in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). This is perhaps not surprising given that ART exposes both oocytes and embryos to high levels of superoxide free radicals during gamete and embryo culture⁶. Indeed, in addition to the culture conditions, *in vitro* the oocytes are not afforded the normal protection of the antioxidant-rich follicular fluid or surrounding cumulus cells, leaving them more susceptible to oxidative damage.^{7,8} Reproductive processes such as chromosome segregation (resulting in euploid oocytes), polar body extrusion (necessary for haploid oocytes), fertilization and early cleavage (to avoid arrested embryos) require energy. As the average age of women seeking fertility treatment rises, the level of ROS and subsequent oxidative stress increases, resulting in mitochondrial DNA mutations which in turn affect ATP production in the oocyte. Without the necessary ATP, required cellular processes cannot occur correctly, having a direct effect on the quality of the oocyte, embryo and the final outcome of the IVF procedure.^{9,10}

It has been shown that oocyte quality begins to deteriorate immediately following ovulation. This has been thought to be an inflammatory driven oxidative stress process¹¹ whereby the production of cytokines and proteases is associated with an increase in ROS which in turn impair oocyte maturation.¹²⁻¹⁴ Evidence of oxidative damage in *in vitro* oocytes has been shown to exist as early as 8 hours after ovulation.¹⁵ Nor surprisingly it has been shown that high levels of the oxidative stress in the follicular fluid of infertile women are associated with poor oocyte maturation and embryo quality, and that inducers of oxidative stress can be used to inhibit oocyte maturation.^{5,12}

1 Together, these observations highlighting the importance of oxidative stress in the developing
2 follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.
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5 Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has
6 important oxygen-scavenging properties which naturally mitigate oxidative stress by both
7 neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.¹⁶⁻¹⁸
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9 Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many
10 medical conditions in which oxidative stress has been implicated including diabetes, glaucoma,
11 irritable bowel syndrome and fertility.¹⁹⁻²²
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14 The effects of melatonin supplementation on culture media,^{12, 21, 23-27} gametes,^{5, 15, 28} embryos^{15,}
15 ^{29, 30} and luteal function³¹⁻³⁵ have been identified as useful areas for investigation. Human clinical
16 studies have begun recently, with emphasis on oral supplementation of melatonin during the
17 stimulation cycle and its effects on oocyte and embryo quality.^{12, 36-39}
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20 Importantly, melatonin has a remarkably benign safety profile in both animal and human studies.
21 A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses
22 at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival,
23 however, also resulted in a reduction in healthy follicles. They concluded that doses of greater
24 than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.⁴⁰ The maternal
25 lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was
26 observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans.
27 Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have
28 been reported for up to doses of 200mg/kg/day.⁴¹ There have been several randomised controlled
29 trials addressing high doses of melatonin in human adults and children,⁴²⁻⁴⁶ and reports have
30 established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.⁴⁷
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39 Rationale

40 In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5
41 cycles resulting in live birth⁴⁸. That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there
42 is much room for further improvement, if only to meet societal expectations and reduce
43 healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF
44 outcomes is therefore merited. Several human and animal studies support the use of melatonin in
45 the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant
46 properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and
47 embryos,⁴⁹ with intrafollicular melatonin concentrations being significantly higher than those
48 found in serum, again suggesting a physiological role in reproduction.^{39, 67, 68} The human studies
49 addressing the use of melatonin in infertility treatment undertaken to date have been small and
50 not placebo-controlled. None have attempted to identify an optimal dose.^{36-38, 50} Further,
51 interpretation of any outcomes have been hampered by the within-patient comparison design,
52 where patients act as their own controls.^{12, 39, 51}
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1 We have designed this study to be the first placebo-controlled, dose-finding clinical trial to
2 investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI
3 treatment.
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8 **Aims**

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10 The overall aim of this trial is to determine whether oral melatonin administration can improve the
11 outcomes of IVF/ICSI. In order to address this, we will determine whether melatonin
12 administration has a dose-response effect in women undergoing IVF/ICSI on:
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- 15 1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-
16 deoxyguanosine (8-OHdg), progesterone, oestradiol)
 - 17 2. sonographic markers of follicle health
 - 18 3. patient sleepiness
 - 19 4. pregnancy rate following IVF/ICSI
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28 **Methods and analyses**

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30 This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.⁵² Recruitment will
31 commence in July 2014. This will occur over two years. Analysis and dissemination will occur after
32 this period of time. The study is expected to be completed by February 2017.
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37 **Study design**

38 Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial
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43 **Subjects**

44 We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.
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50 **Study setting**

51 Patients will be recruited from Monash IVF infertility clinics in Melbourne, Australia over a period
52 of 2 years.
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Interventions to be measured

This trial will have four arms. All capsules will be indistinguishable from each other.

1. Placebo capsule taken twice per day
2. 2mg melatonin capsule twice per day (4mg/d total)
3. 4mg melatonin capsule twice per day (8mg/d total)
4. 8mg melatonin capsule twice per day (16mg/d total)

Primary Outcome

Clinical pregnancy rate,⁵³ defined as the presence of a live pregnancy in the uterine cavity at a transvaginal ultrasound(TVUS) at 6-8 weeks' gestation.

Secondary outcomes

See *Table 1* for details.

Sample Size

As this is a pilot dose-finding study, without precedence on which to base accurate power calculations, a power calculation has not been performed. Part of our aim is to provide clarification allowing for larger randomised trials to be designed in the future. We hypothesise that melatonin will have a positive dose-response effect on clinical pregnancy rates after IVF/ICSI. We have chosen a convenience sample of 160 participants, 40 in each of the four groups, in order to assess plausible morphological, biochemical and sonographic surrogate markers for oocyte quality, as well as to provide an indication of the effect size for each dose for our primary outcome, clinical pregnancy rate. Conservatively estimating that, in total, 50% of patients (n=80) do not get pregnant after their first cycle, we may assume that 60 patients will participate in the crossover arm (allowing for drop-outs) in their second cycle and be allocated to a different treatment group.

Inclusion criteria

1. Undergoing first cycle of IVF or ICSI
2. Age between 18 and 45

3. Body mass index (BMI) between 18 and 35
4. Undergoing a GnRH antagonist cycle (without OCP scheduling)

Exclusion criteria

1. Current untreated pelvic pathology – moderate to severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease, uterine malformations, Asherman's syndrome and hydrosalpinx.
2. Currently enrolled in another interventional clinical trial
3. Concurrent use of other adjuvant therapies (eg. Chinese herbs, acupuncture)
4. Current pregnancy
5. Malignancy or other contraindication to IVF
6. Autoimmune disorders
7. Undergoing preimplantation genetic diagnosis (PGD)
8. Hypersensitivity to melatonin or its metabolites
9. Concurrent use of any of the following medications
 - a. Fluvoxamine (eg. Luvox, Movox, Voxam)
 - b. Cimetidine (eg. Magicul, Tagamet)
 - c. Quinolones and other CYP1A2 inhibitors (Ciprofloxacin, Avalox)
 - d. Carbamazepine (eg. Tegretol), rifampicin (eg. Rifadin) and other CYP1A2 inducers
 - e. Zolpidem (eg. Stilnox), zopiclone (eg. Imovane) and other non-benzodiazepine hypnotics
10. Inability to comply with trial protocol

Concomitant care

Aside from trial protocol and inclusion/exclusion criteria listed above, standard concomitant care, such as folic acid supplements, is permitted.

Participant enrolment

This trial will begin recruitment at the level of the infertility specialist. Once identified as requiring IVF/ICSI, and satisfying inclusion and exclusion criteria, the patient will be provided with detailed written information about the trial protocol. After approximately one week, the patient will be approached and written informed consent will be obtained by the principle investigator at the first visit with the infertility clinic nurse. Baseline blood tests will be taken and basic demographic information including aetiology of infertility will be recorded. The patient will then be randomised by the principle investigator (see below) to one of the study arms and provided with the trial medication. The participant will commence taking the trial medication on the day that ovarian stimulation injections begin (Day 2-3 of menses) at 0800 and 2200 each day, with the last capsule being taken at 2200 the night before oocyte collection.

For a summarised participant timeline, See *Figure 1* and *Figure 2*.

Allocation concealment, blinding and randomisation

Each treatment arm will be randomly allocated a letter (A, B, C or D) by way of opaque sealed envelope. Randomisation will be computerised and patients will be randomised at a ratio of 1:1:1:1 to one of the groups, A-D, using the minimisation method, a method of randomisation accounting for factors known to affect the outcome used in small trials to prevent selection bias.⁵⁴

With a crossover design improved patient recruitment is very likely because all non-pregnant participants will be guaranteed at least one cycle with one of the doses of melatonin.^{55, 56} After completing the first study cycle, those participants that are not pregnant will be recruited for the cross-over cycle in which they will be assigned the next treatment arm. For example, if a participant were allocated to treatment group A in their first cycle and did not become pregnant, they would be offered allocation to group B in their second cycle and so on.

Participants will be blinded by receiving identical-appearing unmarked capsules. All trial researchers will be blinded to treatment allocation group until after analyses are performed at the completion of the trial. A Data Safety Monitoring Committee (see *Adverse events* for details) and dispensing pharmacy will be aware of allocation to allow identification of any unbalanced or multiple serious adverse events that may necessitate emergency unblinding of researchers and participants.

Adherence and retention

In order to ensure the integrity of study data, participant adherence to trial protocol will be assessed on a medication administration record updated daily by the participant. At oocyte collection, participants will return medication bottles and compliance will be confirmed by

1 counting remaining tablets. This will be recorded on individual patient compliance forms and
2 patient record forms. Every reasonable effort will be made from the time of enrolment until the
3 end of follow-up to maintain contact with and maintain subject participation in the trial.
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9 **Analysis plan**

10 SPSS v22.0 (IBM, Armonk, New York) will be used for data analysis. Primary analysis will occur by
11 the intention-to-treat principle. A secondary analysis will be performed for 'actual treatment
12 received'. Statistical analyses will be performed using Chi-square tests for dichotomous categorical
13 outcome variables. Clinical and demographic data will be analysed with parametric tests if they are
14 normally distributed (ANOVA with Bonferroni adjustment), otherwise appropriate non-parametric
15 tests will be implemented (Kruskal-Wallis for between group analyses). Within patient changes
16 (paired data), for example when comparing serum levels of melatonin at baseline and after
17 treatment, will be compared using repeated measures ANOVA or Wilcoxon signed rank test
18 depending on normality of data. $P < 0.05$ will be considered statistically significant. Where
19 statistically sound, adjusted odds ratios will be calculated to account for confounders or effect
20 modifiers. To account for missing data, two analyses will be run, with the first excluding missing
21 values and the second by imputation of missing values to determine how conclusions are affected.
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30 **Adverse events and data safety and monitoring**

31 An independent Data Safety and Monitoring Committee (DSMC) has been established at Monash
32 Health in order to monitor occurrences of adverse events. The principle investigator will be
33 available by telephone at all times during the trial and participants will be provided with contact
34 details in case of any adverse events. Participants will also be interviewed at the time of their
35 ultrasound and the day of oocyte collection to ascertain the occurrence of any adverse events.
36 Serious adverse events will be recorded separately and followed up until resolution. Such events
37 will be reported to the Monash Health, Monash University, Monash Surgical Private Hospital and
38 Epworth HealthCare Human Research Ethics Committees, the Monash Health DSMC, the Monash
39 Health Therapeutics Committee and, if directed by HREC, to the Australian Therapeutics and
40 Goods Administration.
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49 **Trial modification and discontinuation**

50 In the absence of adverse events, the medication regimen will not be modified once commenced.
51 If other protocol changes are deemed necessary by the investigating team, ethics approval will be
52 sought from all approving Human Research Ethics Committees. Once approved, protocol
53 amendments will be notified to all investigators, administrators (eg. dispensing pharmacy) and
54 trial participants via telephone.
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1 Patients are permitted to discontinue their inclusion in the trial at any point in time at their
2 request or following an unexpected serious adverse event as described above. The DSMC will
3 perform an interim analysis specifically assessing adverse events after 50% of participants have
4 been recruited. Otherwise, the trial will cease follow-up once patients have given birth from a trial
5 treatment cycle.
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10 11 **Data collection, informed consent forms and confidentiality**

12 Data will be recorded in both hardcopy and electronic form. Hardcopies will be stored in a secured
13 filing cabinet at the administering institution. Electronic copies will be stored in encrypted files on
14 a password protected computer. All data will be kept for 15 years, following this time, hardcopies
15 will be destroyed by shredding or burning and electronic copies will be deleted by formatting.
16 Organic samples will be stored until analysis in a secured locked temperature-controlled freezer at
17 the Monash Institute of Medical Research that can only be accessed by authorized staff. Samples
18 and participant records will not contain any directly identifiable information. No additional
19 biological samples will be kept for use in ancillary studies.
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25 For access to data collection forms, including sleep diaries, medication compliance and informed
26 consent forms please contact the principle investigator.
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31 **Ethics and dissemination**

32 Ethical approval has been obtained from Monash Health HREC (Ref:13402B), Monash University
33 HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data
34 analysis, interpretation and conclusions will be presented at national and international
35 conferences and published in peer-reviewed journals. De-identified summary results will also be
36 made publically available on the Monash IVF website.
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43 **Discussion**

44 The MIART trial has the potential to improve IVF treatment protocols, with both immediate and
45 translational benefit to patients. If melatonin is found to improve outcomes after IVF/ICSI, it may
46 become a routine part of management of the infertile couple. We aim to set precedence and a
47 framework by which others may structure further investigation into melatonin and other adjuvant
48 therapies in the future. The intention of this study is as a pilot trial, primarily designed to provide
49 un-biased data as a foundation for further research into this area. It is anticipated that the effect
50 size observed in this trial will be useful to more appropriately power subsequent RCTs with the
51 most effective dose of melatonin. In summary, MIART will be the first trial designed to determine
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1 a dose-response relationship of melatonin on biochemical and physiological markers of follicle
2 health and also clinical pregnancy rates.
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5 **Contributors:**

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7 The idea for the trial was conceived by SF, LR, and EW. SF was involved in research design and
8 primary writing of study protocol and manuscript. TO was involved in the design and writing of the
9 manuscript. LR and EW were involved in the trial design, writing, editing and approval of the final
10 manuscript.
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19 in the design, implementation or analysis of this trial.
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23 **Conflict of interest statement**

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25 SF is supported by an NHMRC Postgraduate Scholarship. No conflicts of interest are identified for
26 SF, EW or TO. BV and LR are minority shareholders in Monash IVF. LR has received unconditional
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28 influence on trial design, implementation, analysis or presentation.
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Figure Legends

Figure 1: Participant timeline schematic - first cycle

^aPlacebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin

Table 1: Clinical Secondary Outcomes

Clinical outcomes	Definition
Live birth rate	Birth of a live baby after 24 weeks gestation
Miscarriage rate	Loss of a diagnosed clinical pregnancy before 20 weeks gestation
Sleepiness score	Based on Karolinska ^a sleepiness scale during trial medication administration
Pregnancy complication and adverse events rates	Including OHSS, multiple pregnancy, congenital or chromosomal abnormalities, stillbirth, preeclampsia, delivery before 34 weeks, delivery between 34 and 37 weeks, placenta praevia, gestational diabetes, low birthweight
Embryological outcomes	
Total number of oocytes collected	
Oocyte maturity	
Total number of embryos	
Embryo quality	
Fertilization rate	The proportion of oocytes that become fertilised
Utilisation rate	Proportion of zygotes undergoing embryo transfer or cryopreservation to oocytes fertilised
Biochemical outcomes	
Biochemical pregnancy rate	Presence of serum hCG level of >25IU/l on Day 16 after embryo transfer
Melatonin levels in serum	At baseline and prior to oocyte collection
Melatonin levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
8-OHdg levels in serum	At baseline and prior to oocyte collection
8-OHdg levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
Oestradiol and Progesterone levels in serum	Taken at baseline, during treatment and at the time of oocyte collection
Sonographic outcomes	
Follicular blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection
Uterine artery blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection

OHSS: Ovarian Hyperstimulation syndrome; hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

^aReyner L, Horne J. Falling asleep whilst driving: are drivers aware of prior sleepiness? Int J Legal Med 1998;111:120-3

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A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

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20 **Trial Sponsor:**

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28 **Running Title:** Melatonin in ART - MIART randomised trial: study protocol

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32 **Keywords:**

33 Melatonin, assisted reproductive technology (ART), in-vitro fertilization, oxidative stress, protocol

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38 **Word Count:** 2796

Abstract

Introduction

High levels of oxidative stress can have considerable impact on the outcomes of in-vitro fertilization (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant anti-oxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3-4mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose-response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Methods and analyses

We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2mg twice per day (n=40); melatonin 4mg twice per day (n=40) and melatonin 8mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilization rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy- 2'-deoxyguanosine (8-OHdg) at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores, and pregnancy complications, comparing outcomes between groups. This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.

Ethics and dissemination:

Ethical approval has been obtained from Monash Health HREC (Ref: 13402B), Monash University HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals.

Registration Details: Version 1. Australian New Zealand Clinical Trials Registry, ACTRN: ACTRN12613001317785. The complete WHO Registration Data Set, as described by the SPIRIT 2013 guidelines is available for viewing at <https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=365376>

Strengths and limitations

- This trial is a well-designed pilot study to achieve biochemically and clinically relevant outcomes.
- Significant preliminary research has been performed in order to appropriately design the study to fill gaps in current knowledge.
- This is the first placebo controlled dose-finding trial of melatonin in the field of infertility world-wide and if successful, has the potential to provide a foundation for future large randomised controlled trials.
- Because of constraints of such a pilot study in the infertile population, relatively small numbers of participants are anticipated. To help minimise the effect of this, a cross-over arm will be introduced. It is expected that this limitation will be overcome in future trials that will be designed based on this study.

Introduction

Humans are aerobic organisms and when oxygen is utilised in metabolic processes, free oxygen radicals are created as a consequence. These radicals are oxidants and contain 'free' valence electrons, making them highly unstable and therefore reactive, capable of causing injury to cells. This occurs by accepting electrons from another molecule (a reductant) in order to reach stability. In doing so, the chemical structure of the reductant is changed, and may no longer be able to perform its designated function. In fact, often the reductant can then produce or sometimes even become an oxidant having the potential to cause oxidative stress in its own right.¹ Anti-oxidative agents are present endogenously and can also be administered exogenously. They are designed to reduce free radicals by donating electrons to stabilise the free radical.² The term 'reactive oxygen species' (ROS), not only includes free radicals but also molecularly stable non-radical molecules which contain oxygen and are capable of causing oxidative stress, such as hydrogen peroxide (H₂O₂).³ While ROS are necessary for essential physiological processes, an overabundance can result in cellular and tissue damage and this is commonly referred to as 'oxidative stress'.⁴ Oxidative stress can be measured in bodily fluids by markers including 8-hydroxy- 2'-deoxyguanosine (8-Ohdg).⁵

Over recent years, the potential role of oxidative stress in the outcomes of assisted reproductive technology (ART) has been gaining increasing attention, in particular with regards to in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). This is perhaps not surprising given that ART exposes both oocytes and embryos to high levels of superoxide free radicals during gamete and embryo culture⁶. Indeed, in addition to the culture conditions, *in vitro* the oocytes are not afforded the normal protection of the antioxidant-rich follicular fluid or surrounding cumulus cells, leaving them more susceptible to oxidative damage.^{7,8} Reproductive processes such as chromosome segregation (resulting in euploid oocytes), polar body extrusion (necessary for haploid oocytes), fertilization and early cleavage (to avoid arrested embryos) require energy. As the average age of women seeking fertility treatment rises, the level of ROS and subsequent oxidative stress increases, resulting in mitochondrial DNA mutations which in turn affect ATP production in the oocyte. Without the necessary ATP, required cellular processes cannot occur correctly, having a direct effect on the quality of the oocyte, embryo and the final outcome of the IVF procedure.^{9,10}

It has been shown that oocyte quality begins to deteriorate immediately following ovulation. This has been thought to be an inflammatory driven oxidative stress process¹¹ whereby the production of cytokines and proteases is associated with an increase in ROS which in turn impair oocyte maturation.¹²⁻¹⁴ Evidence of oxidative damage in *in vitro* oocytes has been shown to exist as early as 8 hours after ovulation.¹⁵ Nor surprisingly it has been shown that high levels of the oxidative stress in the follicular fluid of infertile women are associated with poor oocyte maturation and embryo quality, and that inducers of oxidative stress can be used to inhibit oocyte maturation.^{5,12}

1 Together, these observations highlighting the importance of oxidative stress in the developing
2 follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.
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5 Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has
6 important oxygen-scavenging properties which naturally mitigate oxidative stress by both
7 neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.¹⁶⁻¹⁸
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9 Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many
10 medical conditions in which oxidative stress has been implicated including diabetes, glaucoma,
11 irritable bowel syndrome and fertility.¹⁹⁻²²
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14 The effects of melatonin supplementation on culture media,^{12, 21, 23-27} gametes,^{5, 15, 28} embryos^{15,}
15 ^{29, 30} and luteal function³¹⁻³⁵ have been identified as useful areas for investigation. Human clinical
16 studies have begun recently, with emphasis on oral supplementation of melatonin during the
17 stimulation cycle and its effects on oocyte and embryo quality.^{12, 36-39}
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20 Importantly, melatonin has a remarkably benign safety profile in both animal and human studies.
21 A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses
22 at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival,
23 however, also resulted in a reduction in healthy follicles. They concluded that doses of greater
24 than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.⁴⁰ The maternal
25 lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was
26 observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans.
27 Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have
28 been reported for up to doses of 200mg/kg/day.⁴¹ There have been several randomised controlled
29 trials addressing high doses of melatonin in human adults and children,⁴²⁻⁴⁶ and reports have
30 established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.⁴⁷
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39 Rationale

40 In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5
41 cycles resulting in live birth⁴⁸. That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there
42 is much room for further improvement, if only to meet societal expectations and reduce
43 healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF
44 outcomes is therefore merited. Several human and animal studies support the use of melatonin in
45 the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant
46 properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and
47 embryos,⁴⁹ with intrafollicular melatonin concentrations being significantly higher than those
48 found in serum, again suggesting a physiological role in reproduction.^{39, 67, 68} The human studies
49 addressing the use of melatonin in infertility treatment undertaken to date have been small and
50 not placebo-controlled. None have attempted to identify an optimal dose.^{36-38, 50} Further,
51 interpretation of any outcomes have been hampered by the within-patient comparison design,
52 where patients act as their own controls.^{12, 39, 51}
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1 We have designed this study to be the first placebo-controlled, dose-finding clinical trial to
2 investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI
3 treatment.
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8 **Aims**

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10 The overall aim of this trial is to determine whether oral melatonin administration can improve the
11 outcomes of IVF/ICSI. In order to address this, we will determine whether melatonin
12 administration has a dose-response effect in women undergoing IVF/ICSI on:
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- 15 1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-
16 deoxyguanosine (8-OHdg), progesterone, oestradiol)
 - 17 2. sonographic markers of follicle health
 - 18 3. patient sleepiness
 - 19 4. pregnancy rate following IVF/ICSI
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28 **Methods and analyses**

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30 This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.⁵² Recruitment will
31 commence in July 2014. This will occur over two years. Analysis and dissemination will occur after
32 this period of time. The study is expected to be completed by February 2017.
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37 **Study design**

38 Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial
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43 **Subjects**

44 We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.
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50 **Study setting**

51 Patients will be recruited from Monash IVF infertility clinics in Melbourne, Australia over a period
52 of 2 years.
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Interventions to be measured

This trial will have four arms. All capsules will be indistinguishable from each other.

1. Placebo capsule taken twice per day
2. 2mg melatonin capsule twice per day (4mg/d total)
3. 4mg melatonin capsule twice per day (8mg/d total)
4. 8mg melatonin capsule twice per day (16mg/d total)

Primary Outcome

Clinical pregnancy rate,⁵³ defined as the presence of a live pregnancy in the uterine cavity at a transvaginal ultrasound(TVUS) at 6-8 weeks' gestation.

Secondary outcomes

See *Table 1* for details.

Sample Size

As this is a pilot dose-finding study, without precedence on which to base accurate power calculations, a power calculation has not been performed. Part of our aim is to provide clarification allowing for larger randomised trials to be designed in the future. We hypothesise that melatonin will have a positive dose-response effect on clinical pregnancy rates after IVF/ICSI. We have chosen a convenience sample of 160 participants, 40 in each of the four groups, in order to assess plausible morphological, biochemical and sonographic surrogate markers for oocyte quality, as well as to provide an indication of the effect size for each dose for our primary outcome, clinical pregnancy rate. Conservatively estimating that, in total, 50% of patients (n=80) do not get pregnant after their first cycle, we may assume that 60 patients will participate in the crossover arm (allowing for drop-outs) in their second cycle and be allocated to a different treatment group.

Inclusion criteria

1. Undergoing first cycle of IVF or ICSI
2. Age between 18 and 45

3. Body mass index (BMI) between 18 and 35
4. Undergoing a GnRH antagonist cycle (without OCP scheduling)

Exclusion criteria

1. Current untreated pelvic pathology – moderate to severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease, uterine malformations, Asherman's syndrome and hydrosalpinx.
2. Currently enrolled in another interventional clinical trial
3. Concurrent use of other adjuvant therapies (eg. Chinese herbs, acupuncture)
4. Current pregnancy
5. Malignancy or other contraindication to IVF
6. Autoimmune disorders
7. Undergoing preimplantation genetic diagnosis (PGD)
8. Hypersensitivity to melatonin or its metabolites
9. Concurrent use of any of the following medications
 - a. Fluvoxamine (eg. Luvox, Movox, Voxam)
 - b. Cimetidine (eg. Magicul, Tagamet)
 - c. Quinolones and other CYP1A2 inhibitors (Ciprofloxacin, Avalox)
 - d. Carbamazepine (eg. Tegretol), rifampicin (eg. Rifadin) and other CYP1A2 inducers
 - e. Zolpidem (eg. Stilnox), zopiclone (eg. Imovane) and other non-benzodiazepine hypnotics
10. Inability to comply with trial protocol

Concomitant care

Aside from trial protocol and inclusion/exclusion criteria listed above, standard concomitant care, such as folic acid supplements, is permitted.

Participant enrolment

This trial will begin recruitment at the level of the infertility specialist. Once identified as requiring IVF/ICSI, and satisfying inclusion and exclusion criteria, the patient will be provided with detailed written information about the trial protocol. After approximately one week, the patient will be approached and written informed consent will be obtained by the principle investigator at the first visit with the infertility clinic nurse. Baseline blood tests will be taken and basic demographic information including aetiology of infertility will then be taken recorded. ~~and T~~ the patient will will then be randomised by the principle investigator (see below) to one of the study arms and provided with the trial medication. The participant will commence taking the trial medication on the day that ovarian stimulation injections begin (Day 2-3 of menses) at 0800 and 2200 each day, with the last capsule being taken at 2200 the night before oocyte collection.

For a summarised participant timeline, See *Figure 1* and *Figure 2*.

Allocation concealment, blinding and randomisation

Each treatment arm will be randomly allocated a letter (A, B, C or D) by way of opaque sealed envelope. Randomisation will be computerised and patients will be randomised at a ratio of 1:1:1:1 to one of the groups, A-D, using the minimisation method, a method of randomisation accounting for factors known to affect the outcome used in small trials to prevent selection bias.⁵⁴

With a crossover design improved patient recruitment is very likely because all non-pregnant participants will be guaranteed at least one cycle with one of the doses of melatonin.^{55, 56} After completing the first study cycle, those participants that are not pregnant will be recruited for the cross-over cycle in which they will be assigned the next treatment arm. For example, if a participant were allocated to treatment group A in their first cycle and did not become pregnant, they would be offered allocation to group B in their second cycle and so on.

Participants will be blinded by receiving identical-appearing unmarked capsules. All trial researchers will be blinded to treatment allocation group until after analyses are performed at the completion of the trial. A Data Safety Monitoring Committee (see *Adverse events* for details) and dispensing pharmacy will be aware of allocation to allow identification of any unbalanced or multiple serious adverse events that may necessitate emergency unblinding of researchers and participants.

Adherence and retention

In order to ensure the integrity of study data, participant adherence to trial protocol will be assessed on a medication administration record updated daily by the participant. At oocyte collection, participants will return medication bottles and compliance will be confirmed by

1 counting remaining tablets. This will be recorded on individual patient compliance forms and
2 patient record forms. Every reasonable effort will be made from the time of enrolment until the
3 end of follow-up to maintain contact with and maintain subject participation in the trial.
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9 **Analysis plan**

10 SPSS v22.0 (IBM, Armonk, New York) will be used for data analysis. Primary analysis will occur by
11 the intention-to-treat principle. A secondary analysis will be performed for 'actual treatment
12 received'. Statistical analyses will be performed using Chi-square tests for dichotomous categorical
13 outcome variables. Clinical and demographic data will be analysed with parametric tests if they are
14 normally distributed (ANOVA with Bonferroni adjustment), otherwise appropriate non-parametric
15 tests will be implemented (Kruskal-Wallis for between group analyses). Within patient changes
16 (paired data), for example when comparing serum levels of melatonin at baseline and after
17 treatment, will be compared using repeated measures ANOVA or Wilcoxon signed rank test
18 depending on normality of data. $P < 0.05$ will be considered statistically significant. Where
19 statistically sound, adjusted odds ratios will be calculated to account for confounders or effect
20 modifiers. To account for missing data, two analyses will be run, with the first excluding missing
21 values and the second by imputation of missing values to determine how conclusions are affected.
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30 **Adverse events and data safety and monitoring**

31 An independent Data Safety and Monitoring Committee (DSMC) has been established at Monash
32 Health in order to monitor occurrences of adverse events. The principle investigator will be
33 available by telephone at all times during the trial and participants will be provided with contact
34 details in case of any adverse events. Participants will also be interviewed at the time of their
35 ultrasound and the day of oocyte collection to ascertain the occurrence of any adverse events.
36 Serious adverse events will be recorded separately and followed up until resolution. Such events
37 will be reported to the Monash Health, Monash University, Monash Surgical Private Hospital and
38 Epworth HealthCare Human Research Ethics Committees, the Monash Health DSMC, the Monash
39 Health Therapeutics Committee and, if directed by HREC, to the Australian Therapeutics and
40 Goods Administration.
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49 **Trial modification and discontinuation**

50 In the absence of adverse events, the medication regimen will not be modified once commenced.
51 If other protocol changes are deemed necessary by the investigating team, ethics approval will be
52 sought from all approving Human Research Ethics Committees. Once approved, protocol
53 amendments will be notified to all investigators, administrators (eg. dispensing pharmacy) and
54 trial participants via telephone.
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2 Patients are permitted to discontinue their inclusion in the trial at any point in time at their
3 request or following an unexpected serious adverse event as described above. The DSMC will
4 perform an interim analysis specifically assessing adverse events after 50% of participants have
5 been recruited. Otherwise, the trial will cease follow-up once patients have given birth from a trial
6 treatment cycle.
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10 11 **Data collection, informed consent forms and confidentiality**

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13 Data will be recorded in both hardcopy and electronic form. Hardcopies will be stored in a secured
14 filing cabinet at the administering institution. Electronic copies will be stored in encrypted files on
15 a password protected computer. All data will be kept for 15 years, following this time, hardcopies
16 will be destroyed by shredding or burning and electronic copies will be deleted by formatting.
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18 Organic samples will be stored until analysis in a secured locked temperature-controlled freezer at
19 the Monash Institute of Medical Research that can only be accessed by authorized staff. Samples
20 and participant records will not contain any directly identifiable information. No additional
21 biological samples will be kept for use in ancillary studies.
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25 For access to data collection forms, including sleep diaries, medication compliance and informed
26 consent forms please contact the principle investigator.
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30 31 **Ethics and dissemination**

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33 Ethical approval has been obtained from Monash Health HREC (Ref:13402B), Monash University
34 HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data
35 analysis, interpretation and conclusions will be presented at national and international
36 conferences and published in peer-reviewed journals. De-identified summary results will also be
37 made publically available on the Monash IVF website.
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42 43 **Discussion**

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45 The MIART trial has the potential to improve IVF treatment protocols, with both immediate and
46 translational benefit to patients. If melatonin is found to improve outcomes after IVF/ICSI, it may
47 become a routine part of management of the infertile couple. We aim to set precedence and a
48 framework by which others may structure further investigation into melatonin and other adjuvant
49 therapies in the future. The intention of this study is as a pilot trial, primarily designed to provide
50 un-biased data as a foundation for further research into this area. It is anticipated that the effect
51 size observed in this trial will be useful to more appropriately power subsequent RCTs with the
52 most effective dose of melatonin. In summary, MIART will be the first trial designed to determine
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1 a dose-response relationship of melatonin on biochemical and physiological markers of follicle
2 health and also clinical pregnancy rates.
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5 **Contributors:**

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7 The idea for the trial was conceived by SF, LR, and EW. SF was involved in research design and
8 primary writing of study protocol and manuscript. TO was involved in the design and writing of the
9 manuscript. LR and EW were involved in the trial design, writing, editing and approval of the final
10 manuscript.
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14
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19 in the design, implementation or analysis of this trial.
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23 **Conflict of interest statement**

24
25 SF is supported by an NHMRC Postgraduate Scholarship. No conflicts of interest are identified for
26 SF, EW or TO. BV and LR are minority shareholders in Monash IVF. LR has received unconditional
27 research and educational grants from MSD® and Merck Serono®. The trial sponsor had no
28 influence on trial design, implementation, analysis or presentation.
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For peer review only

Figure Legends

Table 1: Clinical Secondary Outcomes

OHSS: Ovarian Hyperstimulation syndrome; hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

^aReyner L, Horne J. Falling asleep whilst driving: are drivers aware of prior sleepiness? Int J Legal Med 1998;111:120-3

Figure 1: Participant timeline schematic - first cycle

^aPlacebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin

Timepoint (days)	Study Period						
	Enrolment	Trial medication begins	Post allocation			Close out	
	-7	0	8-10	14	19	68	299
ENROLMENT							
Eligibility Screen	x						
Informed Consent	x						
Allocation	x						
INTERVENTIONS							
Trial Medication ^a		x	—————	x			
ASSESSMENTS							
Baseline blood tests	x						
Blood tests after treatment				x			
Follicular fluid melatonin and 8-OHdg				x			
Measurement of follicular and uterine blood flow			x				
Oocyte assessments				x			
Embryo assessments				x	—————	x	
Sleepiness scores		x	—————	x			
Pregnancy complication rates and outcomes						x	—————
Followup hCG					x	—————	x

Figure 1: Participant timeline schematic - first cycle

^a Placebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

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Timepoint (days)	Study Period						
	Confirmation of pregnancy failure	Second trial medication begins ^a	8-10	14	19	68	Close out
INTERVENTIONS							
Offer reallocation	x						
Trial Medication ^a		x	-----	x			
ASSESSMENTS							
Oocyte assessments				x			
Embryo assessments				x	-----	x	
Sleepiness scores		x	-----	x			
Pregnancy complication rates and outcomes						x	-----
Followup hCG					x	-----	x

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle
^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)
 ET: embryo transfer; hCG: human chorionic gonadotrophin
 296x419mm (300 x 300 DPI)



STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Page(s)	Section/item	ItemNo	Description
Administrative information			
1	Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym
3	Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry
3		2b	All items from the World Health Organization Trial Registration Data Set
3	Protocol version	3	Date and version identifier
13	Funding	4	Sources and types of financial, material, and other support
1,2,13	Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors
2		5b	Name and contact information for the trial sponsor
13		5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities
11		5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)
Introduction			
5-7	Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention
5-7		6b	Explanation for choice of comparators
7	Objectives	7	Specific objectives or hypotheses
7-11	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)
Methods: Participants, interventions, and outcomes			
7	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained

1	8-9	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)
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4	7-8	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
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8	11		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)
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12	10		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
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16	9		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
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19	8, Table	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended
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26	Fig 1 and	Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)
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31	8	Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations
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35	9-10	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
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43	10	Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions
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50	10	Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
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54	10	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
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58	10	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
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	10		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

Methods: Assignment of interventions (for controlled trials)

Allocation:

Methods: Data collection, management, and analysis

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2	12	Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
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11	10		18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
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15	12	Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
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21	11	Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol
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26	11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
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28	11		20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)
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Methods: Monitoring

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34	11	Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed
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41	11		21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial
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46	11	Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct
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51	11	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
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Ethics and dissemination

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56	12	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
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59	11	Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
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1	10	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
2				
3	N/A		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
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6	12	Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial
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11	13	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site
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14	12	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators
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18	11	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
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21	12	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions
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27	12		31b	Authorship eligibility guidelines and any intended use of professional writers
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29	12		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code
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33		Appendices		
34	Available	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates
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36	request			
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39	12	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable
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*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.

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BMJ Open

A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

Journal:	<i>BMJ Open</i>
Manuscript ID:	bmjopen-2014-005986.R2
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Date Submitted by the Author:	05-Aug-2014
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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine
Keywords:	Reproductive medicine < GYNAECOLOGY, Subfertility < GYNAECOLOGY, Sex steroids & HRT < DIABETES & ENDOCRINOLOGY

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A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

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28 **Running Title:** Melatonin in ART - MIART randomised trial: study protocol

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33 Melatonin, assisted reproductive technology (ART), in-vitro fertilization, oxidative stress, protocol

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Abstract

Introduction

High levels of oxidative stress can have considerable impact on the outcomes of in-vitro fertilization (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant anti-oxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3-4mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose-response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Methods and analyses

We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2mg twice per day (n=40); melatonin 4mg twice per day (n=40) and melatonin 8mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilization rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy- 2'-deoxyguanosine (8-OHdg) at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores, and pregnancy complications, comparing outcomes between groups. This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.

Ethics and dissemination:

Ethical approval has been obtained from Monash Health HREC (Ref: 13402B), Monash University HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals.

Registration Details: Version 1. Australian New Zealand Clinical Trials Registry, ACTRN: ACTRN12613001317785. The complete WHO Registration Data Set, as described by the SPIRIT 2013 guidelines is available for viewing at <https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=365376>

Strengths and limitations

- This trial is a well-designed pilot study to achieve biochemically and clinically relevant outcomes.
- Significant preliminary research has been performed in order to appropriately design the study to fill gaps in current knowledge.
- This is the first randomised placebo controlled dose-finding trial of melatonin in the field of infertility world-wide and if successful, has the potential to provide a foundation for future large randomised controlled trials.
- A cross-over design will be used as this is known to improve recruitment rates

Introduction

Humans are aerobic organisms and when oxygen is utilised in metabolic processes, free oxygen radicals are created as a consequence. These radicals are oxidants and contain 'free' valence electrons, making them highly unstable and therefore reactive, capable of causing injury to cells. This occurs by accepting electrons from another molecule (a reductant) in order to reach stability. In doing so, the chemical structure of the reductant is changed, and may no longer be able to perform its designated function. In fact, often the reductant can then produce or sometimes even become an oxidant having the potential to cause oxidative stress in its own right.¹ Anti-oxidative agents are present endogenously and can also be administered exogenously. They are designed to reduce free radicals by donating electrons to stabilise the free radical.² The term 'reactive oxygen species' (ROS), not only includes free radicals but also molecularly stable non-radical molecules which contain oxygen and are capable of causing oxidative stress, such as hydrogen peroxide (H₂O₂).³ While ROS are necessary for essential physiological processes, an overabundance can result in cellular and tissue damage and this is commonly referred to as 'oxidative stress'.⁴ Oxidative stress can be measured in bodily fluids by markers including 8-hydroxy- 2'-deoxyguanosine (8-Ohdg).⁵

Over recent years, the potential role of oxidative stress in the outcomes of assisted reproductive technology (ART) has been gaining increasing attention, in particular with regards to in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). This is perhaps not surprising given that ART exposes both oocytes and embryos to high levels of superoxide free radicals during gamete and embryo culture⁶. Indeed, in addition to the culture conditions, *in vitro* the oocytes are not afforded the normal protection of the antioxidant-rich follicular fluid or surrounding cumulus cells, leaving them more susceptible to oxidative damage.^{7,8} Reproductive processes such as chromosome segregation (resulting in euploid oocytes), polar body extrusion (necessary for haploid oocytes), fertilization and early cleavage (to avoid arrested embryos) require energy. As the average age of women seeking fertility treatment rises, the level of ROS and subsequent oxidative stress increases, resulting in mitochondrial DNA mutations which in turn affect ATP production in the oocyte. Without the necessary ATP, required cellular processes cannot occur correctly, having a direct effect on the quality of the oocyte, embryo and the final outcome of the IVF procedure.^{9,10}

It has been shown that oocyte quality begins to deteriorate immediately following ovulation. This has been thought to be an inflammatory driven oxidative stress process¹¹ whereby the production of cytokines and proteases is associated with an increase in ROS which in turn impair oocyte maturation.¹²⁻¹⁴ Evidence of oxidative damage in *in vitro* oocytes has been shown to exist as early as 8 hours after ovulation.¹⁵ Nor surprisingly it has been shown that high levels of the oxidative stress in the follicular fluid of infertile women are associated with poor oocyte maturation and embryo quality, and that inducers of oxidative stress can be used to inhibit oocyte maturation.^{5,12}

1 Together, these observations highlighting the importance of oxidative stress in the developing
2 follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.
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5 Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has
6 important oxygen-scavenging properties which naturally mitigate oxidative stress by both
7 neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.¹⁶⁻¹⁸
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9 Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many
10 medical conditions in which oxidative stress has been implicated including diabetes, glaucoma,
11 irritable bowel syndrome and fertility.¹⁹⁻²²
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14 The effects of melatonin supplementation on culture media,^{12, 21, 23-27} gametes,^{5, 15, 28} embryos^{15,}
15 ^{29, 30} and luteal function³¹⁻³⁵ have been identified as useful areas for investigation. Human clinical
16 studies have begun recently, with emphasis on oral supplementation of melatonin during the
17 stimulation cycle and its effects on oocyte and embryo quality.^{12, 36-39}
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20 Importantly, melatonin has a remarkably benign safety profile in both animal and human studies.
21 A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses
22 at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival,
23 however, also resulted in a reduction in healthy follicles. They concluded that doses of greater
24 than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.⁴⁰ The maternal
25 lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was
26 observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans.
27 Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have
28 been reported for up to doses of 200mg/kg/day.⁴¹ There have been several randomised controlled
29 trials addressing high doses of melatonin in human adults and children,⁴²⁻⁴⁶ and reports have
30 established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.⁴⁷
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39 Rationale

40 In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5
41 cycles resulting in live birth⁴⁸. That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there
42 is much room for further improvement, if only to meet societal expectations and reduce
43 healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF
44 outcomes is therefore merited. Several human and animal studies support the use of melatonin in
45 the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant
46 properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and
47 embryos,⁴⁹ with intrafollicular melatonin concentrations being significantly higher than those
48 found in serum, again suggesting a physiological role in reproduction.^{39, 67, 68} The human studies
49 addressing the use of melatonin in infertility treatment undertaken to date have been small and
50 not placebo-controlled. None have attempted to identify an optimal dose.^{36-38, 50} Further,
51 interpretation of any outcomes have been hampered by the within-patient comparison design,
52 where patients act as their own controls.^{12, 39, 51}
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1 We have designed this study to be the first randomised, placebo-controlled, dose-finding clinical
2 trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following
3 IVF/ICSI treatment.
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8 **Aims**

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10 The overall aim of this trial is to determine whether oral melatonin administration can improve the
11 outcomes of IVF/ICSI. In order to address this, we will determine whether melatonin
12 administration has a dose-response effect in women undergoing IVF/ICSI on:
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- 15 1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-
16 deoxyguanosine (8-OHdg), progesterone, oestradiol)
 - 17 2. sonographic markers of follicle health
 - 18 3. patient sleepiness
 - 19 4. pregnancy rate following IVF/ICSI
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28 **Methods and analyses**

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30 This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.⁵² Recruitment will
31 commence in July 2014. This will occur over two years. Analysis and dissemination will occur after
32 this period of time. The study is expected to be completed by February 2017.
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37 **Study design**

38 Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial
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43 **Subjects**

44 We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.
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50 **Study setting**

51 Patients will be recruited from Monash IVF infertility clinics in Melbourne, Australia over a period
52 of 2 years.
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Interventions to be measured

This trial will have four arms. All capsules will be indistinguishable from each other.

1. Placebo capsule taken twice per day
2. 2mg melatonin capsule twice per day (4mg/d total)
3. 4mg melatonin capsule twice per day (8mg/d total)
4. 8mg melatonin capsule twice per day (16mg/d total)

Primary Outcome

Clinical pregnancy rate,⁵³ defined as the presence of a live pregnancy in the uterine cavity at a transvaginal ultrasound (TVUS) at 6-8 weeks' gestation.

Secondary outcomes

See *Table 1* for details.

Sample Size

As this is a pilot dose-finding study, without precedence on which to base accurate power calculations, a power calculation has not been performed. Part of our aim is to provide clarification allowing for larger randomised trials to be designed in the future. We hypothesise that melatonin will have a positive dose-response effect on clinical pregnancy rates after IVF/ICSI. We have chosen a convenience sample of 160 participants, 40 in each of the four groups, in order to assess plausible morphological, biochemical and sonographic surrogate markers for oocyte quality, as well as to provide an indication of the effect size for each dose for our primary outcome, clinical pregnancy rate. Conservatively estimating that, in total, 50% of patients (n=80) do not get pregnant after their first cycle, we may assume that 60 patients will participate in the crossover arm (allowing for drop-outs) in their second cycle and be allocated to a different treatment group.

Inclusion criteria

1. Undergoing first cycle of IVF or ICSI
2. Age between 18 and 45

3. Body mass index (BMI) between 18 and 35
4. Undergoing a GnRH antagonist cycle (without OCP scheduling)

Exclusion criteria

1. Current untreated pelvic pathology – moderate to severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease, uterine malformations, Asherman's syndrome and hydrosalpinx.
2. Currently enrolled in another interventional clinical trial
3. Concurrent use of other adjuvant therapies (eg. Chinese herbs, acupuncture)
4. Current pregnancy
5. Malignancy or other contraindication to IVF
6. Autoimmune disorders
7. Undergoing preimplantation genetic diagnosis (PGD)
8. Hypersensitivity to melatonin or its metabolites
9. Concurrent use of any of the following medications
 - a. Fluvoxamine (eg. Luvox, Movox, Voxam)
 - b. Cimetidine (eg. Magicul, Tagamet)
 - c. Quinolones and other CYP1A2 inhibitors (Ciprofloxacin, Avalox)
 - d. Carbamazepine (eg. Tegretol), rifampicin (eg. Rifadin) and other CYP1A2 inducers
 - e. Zolpidem (eg. Stilnox), zopiclone (eg. Imovane) and other non-benzodiazepine hypnotics
10. Inability to comply with trial protocol

Concomitant care

Aside from trial protocol and inclusion/exclusion criteria listed above, standard concomitant care, such as folic acid supplements, is permitted.

Participant enrolment

This trial will begin recruitment at the level of the infertility specialist. Once identified as requiring IVF/ICSI, and satisfying inclusion and exclusion criteria, the patient will be provided with detailed written information about the trial protocol. After approximately one week, the patient will be approached and written informed consent will be obtained by the principle investigator at the first visit with the infertility clinic nurse. Baseline blood tests will be taken and basic demographic information including aetiology of infertility will be recorded. The patient will then be randomised by the principle investigator (see below) to one of the study arms and provided with the trial medication. The participant will commence taking the trial medication on the day that ovarian stimulation injections begin (Day 2-3 of menses) at 0800 and 2200 each day, with the last capsule being taken at 2200 the night before oocyte collection.

For a summarised participant timeline, See *Figure 1* and *Figure 2*.

Allocation concealment, blinding and randomisation

Association of particular treatment effects (e.g. sleepiness) with certain randomisation codes may become apparent during the trial and to prevent further allocation bias proper concealment of treatment allocation is necessary. Each treatment arm will be randomly allocated a letter (A, B, C or D) by way of opaque sealed envelope. Randomisation will be computerised and patients will be randomised at a ratio of 1:1:1:1 to one of the groups, A-D, using the minimisation method, a method of randomisation accounting for factors known to affect the outcome used in small trials to prevent selection bias.⁵⁴

With a crossover design improved patient recruitment is very likely because all non-pregnant participants will be guaranteed at least one cycle with one of the doses of melatonin.^{55, 56} After completing the first study cycle, those participants that are not pregnant will be recruited for the cross-over cycle in which they will be assigned the next treatment arm. For example, if a participant were allocated to treatment group A in their first cycle and did not become pregnant, they would be offered allocation to group B in their second cycle and so on. The half life of melatonin is short, being completely eliminated from the body shortly after 24 hours.⁵⁷ Once patients have had their first stimulated cycle of treatment, a second stimulated cycle will routinely not commence until at least 4 weeks after the negative pregnancy test (approximately 6 weeks after last melatonin dose), much longer than the elimination time for melatonin. This will be a sufficient washout period prior to inclusion in a second cycle.

Participants will be blinded by receiving identical-appearing unmarked capsules. All trial researchers will be blinded to treatment allocation group until after analyses are performed at the completion of the trial. A Data Safety Monitoring Committee (see *Adverse events* for details) and dispensing pharmacy will be aware of allocation to allow identification of any unbalanced or

1 multiple serious adverse events that may necessitate emergency unblinding of researchers and
2 participants.
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7 **Adherence and retention**

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10 In order to ensure the integrity of study data, participant adherence to trial protocol will be
11 assessed on a medication administration record updated daily by the participant. At oocyte
12 collection, participants will return medication bottles and compliance will be confirmed by
13 counting remaining tablets. This will be recorded on individual patient compliance forms and
14 patient record forms. Every reasonable effort will be made from the time of enrolment until the
15 end of follow-up to maintain contact with and maintain subject participation in the trial.
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19 **Analysis plan**

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22 SPSS v22.0 (IBM, Armonk, New York) will be used for data analysis. Primary analysis will occur by
23 the intention-to-treat principle. A secondary analysis will be performed for 'actual treatment
24 received'. We also intend to perform a separate sub-analysis on the data from the first cycle only.
25 Statistical analyses will be performed using Chi-square tests for dichotomous categorical outcome
26 variables. Clinical and demographic data will be analysed with parametric tests if they are normally
27 distributed (ANOVA with Bonferroni adjustment), otherwise appropriate non-parametric tests will
28 be implemented (Kruskal-Wallis for between group analyses). Within patient changes (paired
29 data), for example when comparing serum levels of melatonin at baseline and after treatment, will
30 be compared using repeated measures ANOVA or Wilcoxon signed rank test depending on
31 normality of data. $P < 0.05$ will be considered statistically significant. Where statistically sound,
32 adjusted odds ratios will be calculated to account for confounders or effect modifiers. To account
33 for missing data, two analyses will be run, with the first excluding missing values and the second
34 by imputation of missing values to determine how conclusions are affected. The SPSS multiple
35 imputation routine (MCMC algorithm known as Fully Conditional Specification) will be used to
36 handle missing data.
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46 **Adverse events and data safety and monitoring**

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48 An independent Data Safety and Monitoring Committee (DSMC) has been established at Monash
49 Health in order to monitor occurrences of adverse events. The principle investigator will be
50 available by telephone at all times during the trial and participants will be provided with contact
51 details in case of any adverse events. Participants will also be interviewed at the time of their
52 ultrasound and the day of oocyte collection to ascertain the occurrence of any adverse events.
53 Serious adverse events will be recorded separately and followed up until resolution. Such events
54 will be reported to the Monash Health, Monash University, Monash Surgical Private Hospital and
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2 Epworth HealthCare Human Research Ethics Committees, the Monash Health DSMC, the Monash
3 Health Therapeutics Committee and, if directed by HREC, to the Australian Therapeutics and
4 Goods Administration.
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8 9 **Trial modification and discontinuation**

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11 In the absence of adverse events, the medication regimen will not be modified once commenced.
12 If other protocol changes are deemed necessary by the investigating team, ethics approval will be
13 sought from all approving Human Research Ethics Committees. Once approved, protocol
14 amendments will be notified to all investigators, administrators (eg. dispensing pharmacy) and
15 trial participants via telephone.
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19 Patients are permitted to discontinue their inclusion in the trial at any point in time at their
20 request or following an unexpected serious adverse event as described above. The DSMC will
21 perform an interim analysis specifically assessing adverse events after 50% of participants have
22 been recruited. Otherwise, the trial will cease follow-up once patients have given birth from a trial
23 treatment cycle.
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28 29 **Data collection, informed consent forms and confidentiality**

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31 Data will be recorded in both hardcopy and electronic form. Hardcopies will be stored in a secured
32 filing cabinet at the administering institution. Electronic copies will be stored in encrypted files on
33 a password protected computer. All data will be kept for 15 years, following this time, hardcopies
34 will be destroyed by shredding or burning and electronic copies will be deleted by formatting.
35 Organic samples will be stored until analysis in a secured locked temperature-controlled freezer at
36 the Monash Institute of Medical Research that can only be accessed by authorized staff. Samples
37 and participant records will not contain any directly identifiable information. No additional
38 biological samples will be kept for use in ancillary studies.
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42 For access to data collection forms, including sleep diaries, medication compliance and informed
43 consent forms please contact the principle investigator.
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48 49 **Ethics and dissemination**

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51 Ethical approval has been obtained from Monash Health HREC (Ref:13402B), Monash University
52 HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data
53 analysis, interpretation and conclusions will be presented at national and international
54 conferences and published in peer-reviewed journals. De-identified summary results will also be
55 made publically available on the Monash IVF website.
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Discussion

The MIART trial has the potential to improve IVF treatment protocols, with both immediate and translational benefit to patients. If melatonin is found to improve outcomes after IVF/ICSI, it may become a routine part of management of the infertile couple. We aim to set precedence and a framework by which others may structure further investigation into melatonin and other adjuvant therapies in the future. The intention of this study is as a pilot trial, primarily designed to provide un-biased data as a foundation for further research into this area. It is anticipated that the effect size observed in this trial will be useful to more appropriately power subsequent RCTs with the most effective dose of melatonin. In summary, MIART will be the first trial designed to determine a dose-response relationship of melatonin on biochemical and physiological markers of follicle health and also clinical pregnancy rates.

Contributors:

The idea for the trial was conceived by SF, LR, and EW. SF was involved in research design and primary writing of study protocol and manuscript. TO was involved in the design and writing of the manuscript. LR and EW were involved in the trial design, writing, editing and approval of the final manuscript.

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Conflict of interest statement

SF is supported by an NHMRC Postgraduate Scholarship. No conflicts of interest are identified for SF, EW or TO. BV and LR are minority shareholders in Monash IVF. LR has received unconditional research and educational grants from MSD® and Merck Serono®. The trial sponsor had no influence on trial design, implementation, analysis or presentation.

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For peer review only

Clinical outcomes	Definition
Live birth rate	Birth of a live baby after 24 weeks gestation
Miscarriage rate	Loss of a diagnosed clinical pregnancy before 20 weeks gestation
Sleepiness score	Based on Karolinska ^a sleepiness scale during trial medication administration
Pregnancy complication and adverse events rates	Including OHSS, multiple pregnancy, congenital or chromosomal abnormalities, stillbirth, preeclampsia, delivery before 34 weeks, delivery between 34 and 37 weeks, placenta praevia, gestational diabetes, low birthweight
Embryological outcomes	
Total number of oocytes collected	
Oocyte maturity	
Total number of embryos	
Embryo quality	
Fertilization rate	The proportion of oocytes that become fertilised
Utilisation rate	Proportion of zygotes undergoing embryo transfer or cryopreservation to oocytes fertilised
Biochemical outcomes	
Biochemical pregnancy rate	Presence of serum hCG level of >25IU/l on Day 16 after embryo transfer
Melatonin levels in serum	At baseline and prior to oocyte collection
Melatonin levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
8-OHdg levels in serum	At baseline and prior to oocyte collection
8-OHdg levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
Oestradiol and Progesterone levels in serum	Taken at baseline, during treatment and at the time of oocyte collection
Sonographic outcomes	
Follicular blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection
Uterine artery blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection

Table 1: Clinical Secondary Outcomes

OHSS: Ovarian Hyperstimulation syndrome; hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy-2'-deoxyguanosine

^aReyner L, Horne J. Falling asleep whilst driving: are drivers aware of prior sleepiness? Int J Legal Med 1998;111:120-3

Figure Legends

Figure 1: Participant timeline schematic - first cycle

^aPlacebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy-2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin

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A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

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28 **Running Title:** Melatonin in ART - MIART randomised trial: study protocol

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32 **Keywords:**

33 Melatonin, assisted reproductive technology (ART), in-vitro fertilization, oxidative stress, protocol

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38 **Word Count:** 2796

Abstract

Introduction

High levels of oxidative stress can have considerable impact on the outcomes of in-vitro fertilization (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant anti-oxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3-4mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose-response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Methods and analyses

We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2mg twice per day (n=40); melatonin 4mg twice per day (n=40) and melatonin 8mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilization rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy- 2'-deoxyguanosine (8-OHdg) at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores, and pregnancy complications, comparing outcomes between groups. This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.

Ethics and dissemination:

Ethical approval has been obtained from Monash Health HREC (Ref: 13402B), Monash University HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals.

Registration Details: Version 1. Australian New Zealand Clinical Trials Registry, ACTRN: ACTRN12613001317785. The complete WHO Registration Data Set, as described by the SPIRIT 2013 guidelines is available for viewing at <https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=365376>

Strengths and limitations

- This trial is a well-designed pilot study to achieve biochemically and clinically relevant outcomes.
- Significant preliminary research has been performed in order to appropriately design the study to fill gaps in current knowledge.
- This is the first randomised placebo controlled dose-finding trial of melatonin in the field of infertility world-wide and if successful, has the potential to provide a foundation for future large randomised controlled trials.

~~— Because of constraints of such a pilot study in the infertile population, relatively small numbers of participants are anticipated. To help minimise the effect of this, a cross-over arm will be introduced. It is expected that this limitation will be overcome in future trials that will be designed based on this study.~~

- A cross-over design will be used as this is known to improve recruitment rates

Introduction

Humans are aerobic organisms and when oxygen is utilised in metabolic processes, free oxygen radicals are created as a consequence. These radicals are oxidants and contain 'free' valence electrons, making them highly unstable and therefore reactive, capable of causing injury to cells. This occurs by accepting electrons from another molecule (a reductant) in order to reach stability. In doing so, the chemical structure of the reductant is changed, and may no longer be able to perform its designated function. In fact, often the reductant can then produce or sometimes even become an oxidant having the potential to cause oxidative stress in its own right.¹ Anti-oxidative agents are present endogenously and can also be administered exogenously. They are designed to reduce free radicals by donating electrons to stabilise the free radical.² The term 'reactive oxygen species' (ROS), not only includes free radicals but also molecularly stable non-radical molecules which contain oxygen and are capable of causing oxidative stress, such as hydrogen peroxide (H₂O₂).³ While ROS are necessary for essential physiological processes, an overabundance can result in cellular and tissue damage and this is commonly referred to as 'oxidative stress'.⁴ Oxidative stress can be measured in bodily fluids by markers including 8-hydroxy- 2'-deoxyguanosine (8-OHdg).⁵

Over recent years, the potential role of oxidative stress in the outcomes of assisted reproductive technology (ART) has been gaining increasing attention, in particular with regards to in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). This is perhaps not surprising given that ART exposes both oocytes and embryos to high levels of superoxide free radicals during gamete and embryo culture⁶. Indeed, in addition to the culture conditions, *in vitro* the oocytes are not afforded the normal protection of the antioxidant-rich follicular fluid or surrounding cumulus cells, leaving them more susceptible to oxidative damage.^{7,8} Reproductive processes such as chromosome segregation (resulting in euploid oocytes), polar body extrusion (necessary for haploid oocytes), fertilization and early cleavage (to avoid arrested embryos) require energy. As the average age of women seeking fertility treatment rises, the level of ROS and subsequent oxidative stress increases, resulting in mitochondrial DNA mutations which in turn affect ATP production in the oocyte. Without the necessary ATP, required cellular processes cannot occur correctly, having a direct effect on the quality of the oocyte, embryo and the final outcome of the IVF procedure.^{9,10}

It has been shown that oocyte quality begins to deteriorate immediately following ovulation. This has been thought to be an inflammatory driven oxidative stress process¹¹ whereby the production of cytokines and proteases is associated with an increase in ROS which in turn impair oocyte maturation.¹²⁻¹⁴ Evidence of oxidative damage in *in vitro* oocytes has been shown to exist as early as 8 hours after ovulation.¹⁵ Nor surprisingly it has been shown that high levels of the oxidative stress in the follicular fluid of infertile women are associated with poor oocyte maturation and embryo quality, and that inducers of oxidative stress can be used to inhibit oocyte maturation.^{5,12}

1 Together, these observations highlighting the importance of oxidative stress in the developing
2 follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.
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5 Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has
6 important oxygen-scavenging properties which naturally mitigate oxidative stress by both
7 neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.¹⁶⁻¹⁸
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9 Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many
10 medical conditions in which oxidative stress has been implicated including diabetes, glaucoma,
11 irritable bowel syndrome and fertility.¹⁹⁻²²
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14 The effects of melatonin supplementation on culture media,^{12, 21, 23-27} gametes,^{5, 15, 28} embryos^{15,}
15 ^{29, 30} and luteal function³¹⁻³⁵ have been identified as useful areas for investigation. Human clinical
16 studies have begun recently, with emphasis on oral supplementation of melatonin during the
17 stimulation cycle and its effects on oocyte and embryo quality.^{12, 36-39}
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20 Importantly, melatonin has a remarkably benign safety profile in both animal and human studies.
21 A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses
22 at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival,
23 however, also resulted in a reduction in healthy follicles. They concluded that doses of greater
24 than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.⁴⁰ The maternal
25 lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was
26 observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans.
27 Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have
28 been reported for up to doses of 200mg/kg/day.⁴¹ There have been several randomised controlled
29 trials addressing high doses of melatonin in human adults and children,⁴²⁻⁴⁶ and reports have
30 established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.⁴⁷
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39 Rationale

40 In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5
41 cycles resulting in live birth⁴⁸. That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there
42 is much room for further improvement, if only to meet societal expectations and reduce
43 healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF
44 outcomes is therefore merited. Several human and animal studies support the use of melatonin in
45 the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant
46 properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and
47 embryos,⁴⁹ with intrafollicular melatonin concentrations being significantly higher than those
48 found in serum, again suggesting a physiological role in reproduction.^{39, 67, 68} The human studies
49 addressing the use of melatonin in infertility treatment undertaken to date have been small and
50 not placebo-controlled. None have attempted to identify an optimal dose.^{36-38, 50} Further,
51 interpretation of any outcomes have been hampered by the within-patient comparison design,
52 where patients act as their own controls.^{12, 39, 51}
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2 | We have designed this study to be the first [randomised](#), placebo-controlled, dose-finding clinical
3 trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following
4 IVF/ICSI treatment.
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8 9 **Aims**

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11 The overall aim of this trial is to determine whether oral melatonin administration can improve the
12 outcomes of IVF/ICSI. In order to address this, we will determine whether melatonin
13 administration has a dose-response effect in women undergoing IVF/ICSI on:
14

- 15 1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-
16 deoxyguanosine (8-OHdg), progesterone, oestradiol)
 - 17 2. sonographic markers of follicle health
 - 18 3. patient sleepiness
 - 19 4. pregnancy rate following IVF/ICSI
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28 29 **Methods and analyses**

30 This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.⁵² Recruitment will
31 commence in July 2014. This will occur over two years. Analysis and dissemination will occur after
32 this period of time. The study is expected to be completed by February 2017.
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37 38 **Study design**

39 Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial
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43 44 **Subjects**

45 We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.
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50 51 **Study setting**

52 Patients will be recruited from Monash IVF infertility clinics in Melbourne, Australia over a period
53 of 2 years.
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Interventions to be measured

This trial will have four arms. All capsules will be indistinguishable from each other.

1. Placebo capsule taken twice per day
2. 2mg melatonin capsule twice per day (4mg/d total)
3. 4mg melatonin capsule twice per day (8mg/d total)
4. 8mg melatonin capsule twice per day (16mg/d total)

Primary Outcome

Clinical pregnancy rate,⁵³ defined as the presence of a live pregnancy in the uterine cavity at a transvaginal ultrasound (TVUS) at 6-8 weeks' gestation.

Secondary outcomes

See *Table 1* for details.

Sample Size

As this is a pilot dose-finding study, without precedence on which to base accurate power calculations, a power calculation has not been performed. Part of our aim is to provide clarification allowing for larger randomised trials to be designed in the future. We hypothesise that melatonin will have a positive dose-response effect on clinical pregnancy rates after IVF/ICSI. We have chosen a convenience sample of 160 participants, 40 in each of the four groups, in order to assess plausible morphological, biochemical and sonographic surrogate markers for oocyte quality, as well as to provide an indication of the effect size for each dose for our primary outcome, clinical pregnancy rate. Conservatively estimating that, in total, 50% of patients (n=80) do not get pregnant after their first cycle, we may assume that 60 patients will participate in the crossover arm (allowing for drop-outs) in their second cycle and be allocated to a different treatment group.

Inclusion criteria

1. Undergoing first cycle of IVF or ICSI
2. Age between 18 and 45

3. Body mass index (BMI) between 18 and 35
4. Undergoing a GnRH antagonist cycle (without OCP scheduling)

Exclusion criteria

1. Current untreated pelvic pathology – moderate to severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease, uterine malformations, Asherman's syndrome and hydrosalpinx.
2. Currently enrolled in another interventional clinical trial
3. Concurrent use of other adjuvant therapies (eg. Chinese herbs, acupuncture)
4. Current pregnancy
5. Malignancy or other contraindication to IVF
6. Autoimmune disorders
7. Undergoing preimplantation genetic diagnosis (PGD)
8. Hypersensitivity to melatonin or its metabolites
9. Concurrent use of any of the following medications
 - a. Fluvoxamine (eg. Luvox, Movox, Voxam)
 - b. Cimetidine (eg. Magicul, Tagamet)
 - c. Quinolones and other CYP1A2 inhibitors (Ciprofloxacin, Avalox)
 - d. Carbamazepine (eg. Tegretol), rifampicin (eg. Rifadin) and other CYP1A2 inducers
 - e. Zolpidem (eg. Stilnox), zopiclone (eg. Imovane) and other non-benzodiazepine hypnotics
10. Inability to comply with trial protocol

Concomitant care

Aside from trial protocol and inclusion/exclusion criteria listed above, standard concomitant care, such as folic acid supplements, is permitted.

Participant enrolment

1 This trial will begin recruitment at the level of the infertility specialist. Once identified as requiring
2 IVF/ICSI, and satisfying inclusion and exclusion criteria, the patient will be provided with detailed
3 written information about the trial protocol. After approximately one week, the patient will be
4 approached and written informed consent will be obtained by the principle investigator at the first
5 visit with the infertility clinic nurse. Baseline blood testss will be taken and basic demographic
6 information including aetiology of infertility will ~~then be taken recorded.~~ ~~and T~~ the patient will will
7 then be randomised by the principle investigator (see below) to one of the study arms and
8 provided with the trial medication. The participant will commence taking the trial medication on
9 the day that ovarian stimulation injections begin (Day 2-3 of menses) at 0800 and 2200 each day,
10 with the last capsule being taken at 2200 the night before oocyte collection.
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16 For a summarised participant timeline, See *Figure 1* and *Figure 2*.
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20 Allocation concealment, blinding and randomisation

21 Association of particular treatment effects (e.g. sleepiness) with certain randomisation codes may
22 become apparent during the trial and to prevent further allocation bias proper concealment of
23 treatment allocation is necessary. Each treatment arm will be randomly allocated a letter (A, B, C
24 or D) by way of opaque sealed envelope. Randomisation will be computerised and patients will be
25 randomised at a ratio of 1:1:1:1 to one of the groups, A-D, using the minimisation method, a
26 method of randomisation accounting for factors known to affect the outcome used in small trials
27 to prevent selection bias.⁵⁴
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33 With a crossover design improved patient recruitment is very likely because all non-pregnant
34 participants will be guaranteed at least one cycle with one of the doses of melatonin.^{55, 56} After
35 completing the first study cycle, those participants that are not pregnant will be recruited for the
36 cross-over cycle in which they will be assigned the next treatment arm. For example, if a
37 participant were allocated to treatment group A in their first cycle and did not become pregnant,
38 they would be offered allocation to group B in their second cycle and so on. The half life of
39 melatonin is short, being completely eliminated from the body shortly after 24 hours.⁵⁷ Once
40 patients have had their first stimulated cycle of treatment, a second stimulated cycle will routinely
41 not commence until at least 4 weeks after the negative pregnancy test (approximately 6 weeks
42 after last melatonin dose), much longer than the elimination time for melatonin. This will be a
43 sufficient washout period prior to inclusion in a second cycle.
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49 Participants will be blinded by receiving identical-appearing unmarked capsules. All trial
50 researchers will be blinded to treatment allocation group until after analyses are performed at the
51 completion of the trial. A Data Safety Monitoring Committee (see *Adverse events* for details) and
52 dispensing pharmacy will be aware of allocation to allow identification of any unbalanced or
53 multiple serious adverse events that may necessitate emergency unblinding of researchers and
54 participants.
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Adherence and retention

In order to ensure the integrity of study data, participant adherence to trial protocol will be assessed on a medication administration record updated daily by the participant. At oocyte collection, participants will return medication bottles and compliance will be confirmed by counting remaining tablets. This will be recorded on individual patient compliance forms and patient record forms. Every reasonable effort will be made from the time of enrolment until the end of follow-up to maintain contact with and maintain subject participation in the trial.

Analysis plan

SPSS v22.0 (IBM, Armonk, New York) will be used for data analysis. Primary analysis will occur by the intention-to-treat principle. A secondary analysis will be performed for 'actual treatment received'. We also intend to perform a separate sub-analysis on the data from the first cycle only. Statistical analyses will be performed using Chi-square tests for dichotomous categorical outcome variables. Clinical and demographic data will be analysed with parametric tests if they are normally distributed (ANOVA with Bonferroni adjustment), otherwise appropriate non-parametric tests will be implemented (Kruskal-Wallis for between group analyses). Within patient changes (paired data), for example when comparing serum levels of melatonin at baseline and after treatment, will be compared using repeated measures ANOVA or Wilcoxon signed rank test depending on normality of data. $P < 0.05$ will be considered statistically significant. Where statistically sound, adjusted odds ratios will be calculated to account for confounders or effect modifiers. To account for missing data, two analyses will be run, with the first excluding missing values and the second by imputation of missing values to determine how conclusions are affected. The SPSS multiple imputation routine (MCMC algorithm known as Fully Conditional Specification) will be used to handle missing data.

Adverse events and data safety and monitoring

An independent Data Safety and Monitoring Committee (DSMC) has been established at Monash Health in order to monitor occurrences of adverse events. The principle investigator will be available by telephone at all times during the trial and participants will be provided with contact details in case of any adverse events. Participants will also be interviewed at the time of their ultrasound and the day of oocyte collection to ascertain the occurrence of any adverse events. Serious adverse events will be recorded separately and followed up until resolution. Such events will be reported to the Monash Health, Monash University, Monash Surgical Private Hospital and Epworth HealthCare Human Research Ethics Committees, the Monash Health DSMC, the Monash

1 Health Therapeutics Committee and, if directed by HREC, to the Australian Therapeutics and
2 Goods Administration.
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7 **Trial modification and discontinuation**

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10 In the absence of adverse events, the medication regimen will not be modified once commenced.
11 If other protocol changes are deemed necessary by the investigating team, ethics approval will be
12 sought from all approving Human Research Ethics Committees. Once approved, protocol
13 amendments will be notified to all investigators, administrators (eg. dispensing pharmacy) and
14 trial participants via telephone.
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17 Patients are permitted to discontinue their inclusion in the trial at any point in time at their
18 request or following an unexpected serious adverse event as described above. The DSMC will
19 perform an interim analysis specifically assessing adverse events after 50% of participants have
20 been recruited. Otherwise, the trial will cease follow-up once patients have given birth from a trial
21 treatment cycle.
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27 **Data collection, informed consent forms and confidentiality**

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29 Data will be recorded in both hardcopy and electronic form. Hardcopies will be stored in a secured
30 filing cabinet at the administering institution. Electronic copies will be stored in encrypted files on
31 a password protected computer. All data will be kept for 15 years, following this time, hardcopies
32 will be destroyed by shredding or burning and electronic copies will be deleted by formatting.
33 Organic samples will be stored until analysis in a secured locked temperature-controlled freezer at
34 the Monash Institute of Medical Research that can only be accessed by authorized staff. Samples
35 and participant records will not contain any directly identifiable information. No additional
36 biological samples will be kept for use in ancillary studies.
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40 For access to data collection forms, including sleep diaries, medication compliance and informed
41 consent forms please contact the principle investigator.
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47 **Ethics and dissemination**

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49 Ethical approval has been obtained from Monash Health HREC (Ref:13402B), Monash University
50 HREC (Ref: CF14/523 – 2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data
51 analysis, interpretation and conclusions will be presented at national and international
52 conferences and published in peer-reviewed journals. De-identified summary results will also be
53 made publically available on the Monash IVF website.
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Discussion

The MIART trial has the potential to improve IVF treatment protocols, with both immediate and translational benefit to patients. If melatonin is found to improve outcomes after IVF/ICSI, it may become a routine part of management of the infertile couple. We aim to set precedence and a framework by which others may structure further investigation into melatonin and other adjuvant therapies in the future. The intention of this study is as a pilot trial, primarily designed to provide un-biased data as a foundation for further research into this area. It is anticipated that the effect size observed in this trial will be useful to more appropriately power subsequent RCTs with the most effective dose of melatonin. In summary, MIART will be the first trial designed to determine a dose-response relationship of melatonin on biochemical and physiological markers of follicle health and also clinical pregnancy rates.

Contributors:

The idea for the trial was conceived by SF, LR, and EW. SF was involved in research design and primary writing of study protocol and manuscript. TO was involved in the design and writing of the manuscript. LR and EW were involved in the trial design, writing, editing and approval of the final manuscript.

Funding

Funding statement: This work is supported both financially and in-kind by the Monash IVF Research and Education Foundation who peer-reviewed this study prior to the approval of funding. In-kind and financial support was also afforded by The Department of Obstetrics and Gynaecology, Monash University and The Ritchie Centre, MIMR-PHI. No funding agency had a role in the design, implementation or analysis of this trial.

Conflict of interest statement

SF is supported by an NHMRC Postgraduate Scholarship. No conflicts of interest are identified for SF, EW or TO. BV and LR are minority shareholders in Monash IVF. LR has received unconditional research and educational grants from MSD® and Merck Serono®. The trial sponsor had no influence on trial design, implementation, analysis or presentation.

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Figure Legends

Table 1: Clinical Secondary Outcomes

OHSS: Ovarian Hyperstimulation syndrome; hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

^aReyner L, Horne J. Falling asleep whilst driving: are drivers aware of prior sleepiness? Int J Legal Med 1998;111:120-3

Figure 1: Participant timeline schematic - first cycle

^aPlacebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin

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	Study Period							
	Enrolment	Trial medication begins	Post allocation			Close out		
Timepoint (days)	-7	0	8-10	14	19	68	299	
ENROLMENT								
Eligibility Screen	x							
Informed Consent	x							
Allocation	x							
INTERVENTIONS								
Trial Medication ^a		x	—————	x				
ASSESSMENTS								
Baseline blood tests	x							
Blood tests after treatment				x				
Follicular fluid melatonin and 8-OHdg				x				
Measurement of follicular and uterine blood flow			x					
Oocyte assessments				x				
Embryo assessments				x	—————	x		
Sleepiness scores		x	—————	x				
Pregnancy complication rates and outcomes						x	—————	x
Followup hCG					x	—————	x	

Figure 1: Participant timeline schematic - first cycle

^a Placebo, melatonin 2mg twice daily, melatonin 4mg twice daily, melatonin 8mg twice daily

hCG: human chorionic gonadotrophin; 8-OHdg: 8-hydroxy- 2'-deoxyguanosine

Note: Patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (Figure 2)

296x419mm (300 x 300 DPI)

Timepoint (days)	Study Period							
	Confirmation of pregnancy failure	Second trial medication begins ^a	Post reallocation			Close out		
		0	8-10	14	19	68	299	
INTERVENTIONS								
Offer reallocation	x							
Trial Medication ^a		x	—————	x				
ASSESSMENTS								
Oocyte assessments				x				
Embryo assessments				x	———	x		
Sleepiness scores		x	—————	x				
Pregnancy complication rates and outcomes						x	—————	x
Followup hCG					x	———	x	

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle

^a For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and D to A)

ET: embryo transfer; hCG: human chorionic gonadotrophin

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STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Page(s)	Section/item	ItemNo	Description
Administrative information			
1	Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym
3	Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry
3		2b	All items from the World Health Organization Trial Registration Data Set
3	Protocol version	3	Date and version identifier
13	Funding	4	Sources and types of financial, material, and other support
1,2,13	Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors
2		5b	Name and contact information for the trial sponsor
13		5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities
11		5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)
Introduction			
5-7	Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention
5-7		6b	Explanation for choice of comparators
7	Objectives	7	Specific objectives or hypotheses
7-11	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)
Methods: Participants, interventions, and outcomes			
7	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained

1	8-9	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)
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4	7-8	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
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8	11		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)
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12	10		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
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16	9		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
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19	8, Table	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended
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26	Fig 1 and	Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)
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31	8	Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations
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35	9-10	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
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Methods: Assignment of interventions (for controlled trials)

Allocation:

43	10	Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions
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50	10	Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
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54	10	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
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58	10	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
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	10		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

Methods: Data collection, management, and analysis

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2	12	Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
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11	10		18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
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15	12	Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
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21	11	Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol
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26	11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
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28	11		20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)
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Methods: Monitoring

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34	11	Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed
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41	11		21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial
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46	11	Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct
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51	11	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
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Ethics and dissemination

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56	12	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
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59	11	Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
60				

10	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
N/A		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
12	Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial
13	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site
12	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators
11	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
12	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions
12		31b	Authorship eligibility guidelines and any intended use of professional writers
12		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code
Appendices			
Available on request	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates
12	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.