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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 culture[6] . 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 process[11] 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]

Together, these observations highlighting the importance of oxidative stress in the developing follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.

Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has important oxygen-scavenging properties which naturally mitigate oxidative stress by both neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.[16-18] Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many medical conditions in which oxidative stress has been implicated including diabetes, glaucoma, irritable bowel syndrome and fertility.[19-22]

The effects of melatonin supplementation on culture media, [12, 21, 23-27] gametes, [5, 15, 28] embryos[15, 29, 30] and luteal function[31-35] have been identified as useful areas for investigation. Human clinical studies have begun recently, with emphasis on oral supplementation of melatonin during the stimulation cycle and its effects on oocyte and embryo quality. [12, 36-39]

Importantly, melatonin has a remarkably benign safety profile in both animal and human studies. A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival, however, also resulted in a reduction in healthy follicles. They concluded that doses of greater than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.[40] The maternal lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans. Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have been reported for up to doses of 200mg/kg/day.[41] There have been several randomised controlled trials addressing high doses of melatonin in human adults and children, [42-46] and reports have established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.[47]

Rationale

In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5 cycles resulting in live birth[48] . That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there is much room for further improvement, if only to meet societal expectations and reduce healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF outcomes is therefore merited. Several human and animal studies support the use of melatonin in the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and embryos,[49] with intrafollicular melatonin concentrations being significantly higher than those found in serum, again suggesting a physiological role in reproduction. [39, 67, 68] The human studies addressing the use of melatonin in infertility treatment undertaken to date have been small and not placebo-controlled. None have attempted to identify an optimal dose. [36-38, 50] Further, interpretation of any outcomes have been hampered by the within-patient comparison design, where patients act as their own controls. [12, 39, 51]

We have designed this study to be the first placebo-controlled, dose-finding clinical trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI treatment.

Aims

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

1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-deoxyguanosine (8-OHdg), progesterone, oestradiol)
2. sonographic markers of follicle health
3. patient sleepiness
4. pregnancy rate following IVF/ICSI

Methods and analyses

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

Study design

Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial

Subjects

We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.

Study setting

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

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. Carbemazepine (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 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

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

[a]Reyner 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

[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)

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

[a]Reyner 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

[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*)

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

SPIRIT

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

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)
7-8	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
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)
10		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
9		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
8, Table 1	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
Fig 1 and 2	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)
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
9-10	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
	Methods: Assignment of interventions (for controlled trials)		
	Allocation:		
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
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
10	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
10	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
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		
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
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
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
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
11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
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)
	Methods: Monitoring		
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
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
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
11	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
	Ethics and dissemination		
12	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
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)

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" license.

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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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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 culture[6] . 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 process[11] 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]

Together, these observations highlighting the importance of oxidative stress in the developing follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.

Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has important oxygen-scavenging properties which naturally mitigate oxidative stress by both neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.[16-18] Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many medical conditions in which oxidative stress has been implicated including diabetes, glaucoma, irritable bowel syndrome and fertility.[19-22]

The effects of melatonin supplementation on culture media, [12, 21, 23-27] gametes, [5, 15, 28] embryos[15, 29, 30] and luteal function[31-35] have been identified as useful areas for investigation. Human clinical studies have begun recently, with emphasis on oral supplementation of melatonin during the stimulation cycle and its effects on oocyte and embryo quality. [12, 36-39]

Importantly, melatonin has a remarkably benign safety profile in both animal and human studies. A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival, however, also resulted in a reduction in healthy follicles. They concluded that doses of greater than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.[40] The maternal lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans. Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have been reported for up to doses of 200mg/kg/day.[41] There have been several randomised controlled trials addressing high doses of melatonin in human adults and children, [42-46] and reports have established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.[47]

Rationale

In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5 cycles resulting in live birth[48] . That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there is much room for further improvement, if only to meet societal expectations and reduce healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF outcomes is therefore merited. Several human and animal studies support the use of melatonin in the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and embryos,[49] with intrafollicular melatonin concentrations being significantly higher than those found in serum, again suggesting a physiological role in reproduction. [39, 67, 68] The human studies addressing the use of melatonin in infertility treatment undertaken to date have been small and not placebo-controlled. None have attempted to identify an optimal dose. [36-38, 50] Further, interpretation of any outcomes have been hampered by the within-patient comparison design, where patients act as their own controls. [12, 39, 51]

We have designed this study to be the first placebo-controlled, dose-finding clinical trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI treatment.

Aims

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

1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-deoxyguanosine (8-OHdg), progesterone, oestradiol)
2. sonographic markers of follicle health
3. patient sleepiness
4. pregnancy rate following IVF/ICSI

Methods and analyses

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

Study design

Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial

Subjects

We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.

Study setting

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

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. Carbemazepine (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

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 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

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)

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

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

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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Running Title: Melatonin in ART - MIART randomised trial: 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 culture[6] . 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 process[11] 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]

Together, these observations highlighting the importance of oxidative stress in the developing follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.

Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has important oxygen-scavenging properties which naturally mitigate oxidative stress by both neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.[16-18] Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many medical conditions in which oxidative stress has been implicated including diabetes, glaucoma, irritable bowel syndrome and fertility.[19-22]

The effects of melatonin supplementation on culture media, [12, 21, 23-27] gametes, [5, 15, 28] embryos[15, 29, 30] and luteal function[31-35] have been identified as useful areas for investigation. Human clinical studies have begun recently, with emphasis on oral supplementation of melatonin during the stimulation cycle and its effects on oocyte and embryo quality. [12, 36-39]

Importantly, melatonin has a remarkably benign safety profile in both animal and human studies. A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival, however, also resulted in a reduction in healthy follicles. They concluded that doses of greater than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.[40] The maternal lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans. Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have been reported for up to doses of 200mg/kg/day.[41] There have been several randomised controlled trials addressing high doses of melatonin in human adults and children, [42-46] and reports have established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.[47]

Rationale

In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5 cycles resulting in live birth[48] . That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there is much room for further improvement, if only to meet societal expectations and reduce healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF outcomes is therefore merited. Several human and animal studies support the use of melatonin in the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and embryos,[49] with intrafollicular melatonin concentrations being significantly higher than those found in serum, again suggesting a physiological role in reproduction. [39, 67, 68] The human studies addressing the use of melatonin in infertility treatment undertaken to date have been small and not placebo-controlled. None have attempted to identify an optimal dose. [36-38, 50] Further, interpretation of any outcomes have been hampered by the within-patient comparison design, where patients act as their own controls. [12, 39, 51]

We have designed this study to be the first placebo-controlled, dose-finding clinical trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI treatment.

Aims

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

1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-deoxyguanosine (8-OHdg), progesterone, oestradiol)
2. sonographic markers of follicle health
3. patient sleepiness
4. pregnancy rate following IVF/ICSI

Methods and analyses

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

Study design

Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial

Subjects

We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.

Study setting

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

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. Carbemazepine (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~~s~~ will be taken and basic demographic information including aetiology of infertility will ~~then~~ be ~~taken~~recorded. ~~and~~ T~~t~~he 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

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 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

[a]Reyner 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

[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)

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

	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	

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)

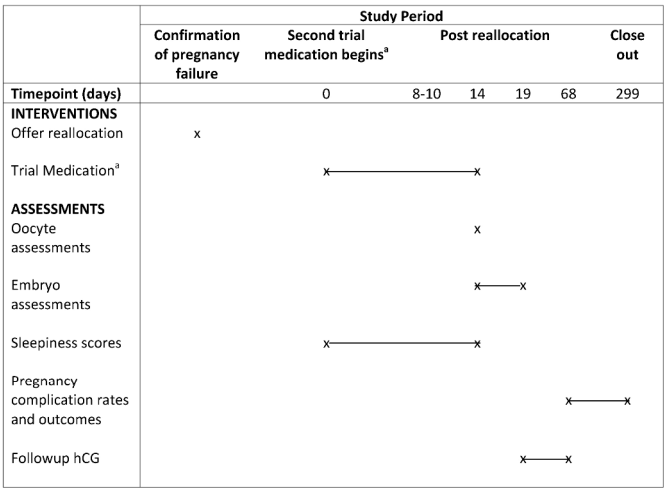

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle
[a] For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and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

SPIRIT

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

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)
7-8	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
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)
10		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
9		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
8, Table 1	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
Fig 1 and 2	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)
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
9-10	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
	Methods: Assignment of interventions (for controlled trials)		
	Allocation:		
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
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
10	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
10	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
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		
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
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
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
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
11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
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)
	Methods: Monitoring		
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
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
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
11	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
	Ethics and dissemination		
12	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
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)

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" license.

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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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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 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 culture[6] . 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 process[11] 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]

Together, these observations highlighting the importance of oxidative stress in the developing follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.

Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has important oxygen-scavenging properties which naturally mitigate oxidative stress by both neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.[16-18] Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many medical conditions in which oxidative stress has been implicated including diabetes, glaucoma, irritable bowel syndrome and fertility.[19-22]

The effects of melatonin supplementation on culture media, [12, 21, 23-27] gametes, [5, 15, 28] embryos[15, 29, 30] and luteal function[31-35] have been identified as useful areas for investigation. Human clinical studies have begun recently, with emphasis on oral supplementation of melatonin during the stimulation cycle and its effects on oocyte and embryo quality. [12, 36-39]

Importantly, melatonin has a remarkably benign safety profile in both animal and human studies. A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival, however, also resulted in a reduction in healthy follicles. They concluded that doses of greater than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.[40] The maternal lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans. Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have been reported for up to doses of 200mg/kg/day.[41] There have been several randomised controlled trials addressing high doses of melatonin in human adults and children, [42-46] and reports have established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.[47]

Rationale

In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5 cycles resulting in live birth[48] . That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there is much room for further improvement, if only to meet societal expectations and reduce healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF outcomes is therefore merited. Several human and animal studies support the use of melatonin in the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and embryos,[49] with intrafollicular melatonin concentrations being significantly higher than those found in serum, again suggesting a physiological role in reproduction. [39, 67, 68] The human studies addressing the use of melatonin in infertility treatment undertaken to date have been small and not placebo-controlled. None have attempted to identify an optimal dose. [36-38, 50] Further, interpretation of any outcomes have been hampered by the within-patient comparison design, where patients act as their own controls. [12, 39, 51]

We have designed this study to be the first randomised, placebo-controlled, dose-finding clinical trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI treatment.

Aims

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

1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-deoxyguanosine (8-OHdg), progesterone, oestradiol)
2. sonographic markers of follicle health
3. patient sleepiness
4. pregnancy rate following IVF/ICSI

Methods and analyses

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

Study design

Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial

Subjects

We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.

Study setting

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

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. Carbemazepine (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

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'. 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 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 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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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

[a]Reyner 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

[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)

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

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 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 culture[6] . 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 process[11] 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]

Together, these observations highlighting the importance of oxidative stress in the developing follicle and in the future success of fertilization, whether *in vivo* or *in vitro*.

Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has important oxygen-scavenging properties which naturally mitigate oxidative stress by both neutralising ROS in human tissues and by inducing endogenous anti-oxidant enzymes.[16-18] Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many medical conditions in which oxidative stress has been implicated including diabetes, glaucoma, irritable bowel syndrome and fertility.[19-22]

The effects of melatonin supplementation on culture media, [12, 21, 23-27] gametes, [5, 15, 28] embryos[15, 29, 30] and luteal function[31-35] have been identified as useful areas for investigation. Human clinical studies have begun recently, with emphasis on oral supplementation of melatonin during the stimulation cycle and its effects on oocyte and embryo quality. [12, 36-39]

Importantly, melatonin has a remarkably benign safety profile in both animal and human studies. A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses at 200mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival, however, also resulted in a reduction in healthy follicles. They concluded that doses of greater than 20mg/kg/day resulted in a reduction in both healthy follicles and ovarian size.[40] The maternal lowest observed adverse effect level (LOAEL), the lowest level at which an adverse event was observed, appears to be around 200mg/kg/day, equivalent to over 1g per day for humans. Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have been reported for up to doses of 200mg/kg/day.[41] There have been several randomised controlled trials addressing high doses of melatonin in human adults and children, [42-46] and reports have established that toxicity levels in humans are not reached until a daily dose of at least 5mg/kg.[47]

Rationale

In the USA in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5 cycles resulting in live birth[48] . That is 4 out of 5 IVF cycles do not result in a live baby. Clearly there is much room for further improvement, if only to meet societal expectations and reduce healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF outcomes is therefore merited. Several human and animal studies support the use of melatonin in the treatment of infertility, with beneficial actions being largely attributed to its anti-oxidant properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and embryos,[49] with intrafollicular melatonin concentrations being significantly higher than those found in serum, again suggesting a physiological role in reproduction. [39, 67, 68] The human studies addressing the use of melatonin in infertility treatment undertaken to date have been small and not placebo-controlled. None have attempted to identify an optimal dose. [36-38, 50] Further, interpretation of any outcomes have been hampered by the within-patient comparison design, where patients act as their own controls. [12, 39, 51]

We have designed this study to be the first randomised, placebo-controlled, dose-finding clinical trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI treatment.

Aims

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

1. biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-deoxyguanosine (8-OHdg), progesterone, oestradiol)
2. sonographic markers of follicle health
3. patient sleepiness
4. pregnancy rate following IVF/ICSI

Methods and analyses

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

Study design

Pilot phase II cross-over double-blinded randomised placebo-controlled dose-response trial

Subjects

We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.

Study setting

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

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. Carbemazepine (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~~s~~ will be taken and basic demographic information including aetiology of infertility will ~~then~~ be ~~taken~~recorded. ~~and~~ T~~t~~he 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

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 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'. 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

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 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

[a]Reyner 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

[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)

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

	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	

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)

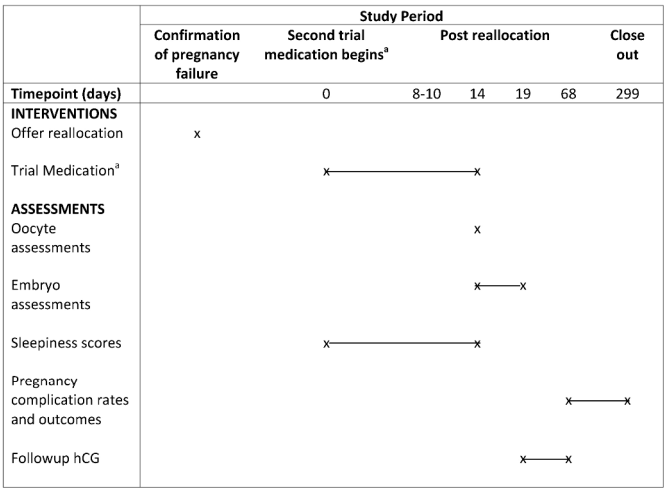

Figure 2: Participant timeline schematic - second cycle in those not pregnant after their first cycle
[a] For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (ie. A will be allocated to B, B to C, C to D and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

SPIRIT
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

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)
7-8	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
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)
10		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
9		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
8, Table 1	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
Fig 1 and 2	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)
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
9-10	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
	Methods: Assignment of interventions (for controlled trials)		
	Allocation:		
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
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
10	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
10	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
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		
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
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
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
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
11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
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)
	Methods: Monitoring		
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
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
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
11	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
	Ethics and dissemination		
12	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
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)

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" license.