A Case Control Study of Melatonin with or Without Coenzyme Q10 in Improving Oocyte Quality and Outcomes in In vitro Fertilization

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ABSTRACT: Antioxidant therapy is thought to improve oocyte quality through the reduction of oxidative stress. Melatonin and coenzyme Q10 (CoQ10) are known antioxidants. We aimed to investigate the clinical efficacy of exogenous melatonin supplementation with or without CoQ10 in improving oocyte quantity and quality. Fifty-three women undergoing cycles at Monash IVF were supplemented with melatonin 4 mg daily, with 19 of those additionally supplemented with 150 mg of CoQ10. Controls were matched at a 2:1 ratio to cases. Data were taken from an index (pretherapy) cycle and from a therapeutic (posttherapy) cycle for both cases and controls. Comparisons were made directly between cases and controls in the therapeutic cycle and by comparing the median differences of the change in outcomes from the index to therapeutic cycle. Primary outcomes included follicle, oocyte, and metaphase II oocytes (MII) counts. Results showed higher median absolute oocyte numbers (6.00 vs. 4.00, $P = 0.049$) in the case group. Cases and controls were significantly different when comparing median change between cycles in oocyte number (0.00 vs. −1.00, $P = 0.028$) and MII count (0.00 vs. −1.00, $P = 0.034$). However, this was the result of poorer outcomes in the control group rather than improvements in the case group, and it cannot be concluded that treatment improved oocyte quality.

KEYWORDS: melatonin, coenzyme Q10, oxidative stress, mitochondrial dysfunction

Introduction

Poor oocyte quality is a limiting factor in achieving successful live births through in vitro fertilization (IVF). Oocyte donation leading to reproductive success even in late–maternal age women suggests that if oocyte quality can be improved, then successful pregnancies can occur. This has led to a recent focus on finding effective techniques to improve the quality of the oocyte.

Oocyte development is complex and energy-intensive, requiring high levels of functional mitochondria to produce sufficient adenosine triphosphate (ATP) for the meiotic process.[1] Insufficient or poorly functioning mitochondria lead to reduced energy levels, which in turn can lead to arrested development of the oocyte,[2] increased aneuploidy rates,[3] and decreased fertilization rates.[4]

The process of aerobic metabolism produces reactive oxygen species (ROS), which are a natural and important part of many physiological processes. ROS is a generic term incorporating all oxygen-containing molecules that are chemically reactive. This includes both reactive molecules with a stable chemical structure and *free radicals* or molecules with unpaired valence electrons that make them unstable and highly reactive.[5] Usually, the ROS are kept in balance by endogenous antioxidants and antioxidant enzymes, which inhibit the oxidation of molecules and prevent the formation of free radicals or act as reducing agents and revert the ROS to their original form. If this balance is disrupted and there becomes an overabundance of ROS, a state of oxidative stress can occur.[6]

Oxidative stress can be detrimental to the reproductive pathway for many reasons, one being genetic damage to both cellular and mitochondrial DNA.[7] Mitochondria are particularly susceptible to damage, in part due to their proximity to the source of ROS generation.[8,9] If mitochondria become damaged, it can create a self-perpetuating cycle as defective respiration leads to a further increase in ROS. With damaged mitochondria, the energy-intensive processes of meiosis, fertilization, and blastocyst formation can become impaired. Studies have indicated that oxidative stress reduces the quality of the oocytes and embryos,[10,11] fertilization rates, and, most importantly, the overall success rates of IVF treatments.[12]

On the other hand, higher antioxidant levels in the follicular fluid of infertility patients were associated with successful pregnancies.[13]

Many patient factors present in an infertile population, such as late maternal age or extremes of body weight, are independently associated with higher levels of oxidative stress.[14] The IVF process itself can cause exposure to high levels of free radicals even before oocyte retrieval.[15] Retrieval of oocytes from the ovaries then removes the protective effect of the endogenous antioxidants present in the follicular fluid.[16]

In a state of oxidative stress, exogenous administration of antioxidants should theoretically improve mitochondrial function. However, a recent Cochrane review on antioxidants in female subfertility could draw no useful conclusions based on the quality of the evidence; thus, further research is required.[17]

Melatonin. Melatonin is an endogenously produced indoleamine derived from 5-hydroxytryptamine. Melatonin has many physiological functions and is well known for its role in the regulation of circadian rhythms.[18] However, its most important role in the infertility setting may be as an antioxidant.[19]

Melatonin is a broad-spectrum antioxidant and free radical scavenger of ROS, which can be enhanced by pharmacological administration.[20] Melatonin produces many metabolites that are by themselves effective free radical scavengers and additionally enhance the actions of antioxidant enzymes and other antioxidants, such as glutathione peroxidase and superoxide dismutase.[21,22] Thus, melatonin creates a cascade reaction of antioxidant protection. The protective effect of melatonin has been studied and documented in other medical diseases associated with oxidative stress, such as Alzheimer's disease[23] and cardiac reperfusion injuries.[24] Endogenous melatonin concentrations naturally decrease with age.[25]

Although preliminary trials have suggested that melatonin may improve IVF outcomes, a recent systematic review and meta-analysis on all randomized controlled trials involving melatonin[26–30] could not show a definite benefit due to the poor quality evidence.[31]

Melatonin has an optimal therapeutic window, below which has no clinical effect and above which can be toxic to the oocyte. This has been demonstrated in multiple animal studies[32–35] as well as with human oocytes in culture medium.[36] For clinical purposes, exogenous supplementation of 3 mg significantly increased follicular fluid melatonin concentrations.[29,37] Melatonin at this dose is safe for short-term use[17] and is naturally present in the fetal circulation.[38]

Coenzyme Q10. Coenzyme Q10 (CoQ10) is an endogenously produced component of cell membranes that plays an important role in the respiratory cycle.[39] CoQ10 aids in the ejection of protons into the intermembrane space, creating an electrical and chemical gradient that enables the phosphorylation of adenosine diphosphate to ATP.[40] During production of the electrical gradient, CoQ10 becomes reduced,

allowing it to act as an ROS scavenger itself.[41] If there is a CoQ10 deficiency, this process is disrupted, free radicals accumulate, and oxidative stress occurs.[42] The positive antioxidant effects of CoQ10 have been demonstrated in multiple medical conditions associated with mitochondrial dysfunction including congestive cardiac failure,[43] Parkinson's disease,[44] and asthenozoospermia.[45]

CoQ10 concentrations decline with age,[46] leading to increased ROS production, lipid oxidation, and cell necrosis.[47] Higher follicular fluid CoQ10 concentrations have been correlated with high-grade embryos, whereas CoQ10 deficiencies have been associated with dysmorphic oocytes.[48] The sole prospective trial of CoQ10 in isolation was of 39 women treated with CoQ10 and did not show a significant increase in top quality embryos (64.7% in the case group vs. 42% in the control group); however, the study was underpowered as it was terminated early.[49]

If deficient in CoQ10, dietary supplementation with doses of 100–3,000 mg/day have shown significant plasma increases and are safe for short-term use.[50] CoQ10 is naturally present in the fetal circulation.[51]

We hypothesized that exogenous supplementation of melatonin with or without the addition of CoQ10 would improve the oocyte quantity and quality for women at risk of oxidative stress. We aimed to assess whether exogenous supplementation of 4 mg of melatonin, with or without the addition of 150 mg of CoQ10, could improve IVF outcomes.

Materials and Methods

Study design. The study was performed as a retrospective case control study conducted by Monash IVF at multiple sites in Victoria. Institutional review board approval was obtained from the Monash Surgical Private Hospital (ethics approval number 07078).

Inclusion/exclusion criteria. Fifty-three women (31–44 years old) with no major systemic illnesses were included in the study. Thirty-four women were given melatonin 4 mg oral daily from on or before the first day of follicle stimulation. Nineteen women in addition to melatonin received 150 mg of CoQ10 from at least 1 month prior to the commencement of the IVF cycle. All women had undergone at least one unsuccessful cycle at Monash IVF without antioxidant therapy, followed by a consecutive stimulation cycle after commencement of antioxidant therapy. The two cycles were identical in stimulation type and follicle stimulating hormone (FSH) dose, and within 1 year of the other.

These women were taken from a potential pool of 441 women on melatonin or CoQ10 who were being treated by one of two reproductive endocrinology and infertility specialists. The decision to commence the women on antioxidant therapy was at the discretion of the two fertility specialists. The women's treatment records were accessed and any woman on other complementary therapies known or suspected to affect oocyte quality were excluded. Women were also excluded if

the cycle after commencing antioxidant therapy was different in protocol to the pretreatment cycle, or if they had a period of greater than 1 year between them. All cycles occurred between September 2011 and October 2013.

Other studies had used 3 mg of melatonin; however, this dosage was not available in Australia, and 4 mg was used as the closest pharmacological dosage available. At the time of the study, CoQ10 for human female infertility had not been studied and the dosage of 150 mg was chosen based on a commonly administered dose for treatment of cardiac failure.[43] Ten women (18%) were commenced on melatonin three months prior to their IVF cycle, while the remainder were started on the day of follicle stimulation.

IVF therapy. Antagonist, downregulation, or boost cycles were conducted according to the Monash IVF treatment protocols. In antagonist cycles, FSH stimulation was commenced 2 days after a spontaneous or oral contraceptive pill (OCP)–induced bleed. In downregulation cycles, the gonadotropin-releasing hormone agonist (GnRHa) was commenced in the mid-luteal phase of a previous spontaneous cycle or day 17 of an OCP cycle, with FSH given once downregulation was confirmed with estradiol (E2) concentrations of ≤200 pmol/L. In a boost cycle, GnRHa was given on days 1–2 after a spontaneous or OCP-induced bleed, with FSH commencing the following day.

Once started, an uninterrupted IVF cycle was performed unless the cycle was canceled at the discretion of the clinician in conjunction with the patient due to inadequate follicle number. Oocyte retrieval was performed 38 hours after human chorionic gonadotropin (hCG) administration. Both intracytoplasmic sperm injection and standard inseminations (standard IVF) were performed. Fertilization results were assessed 16–20 hours postinsemination. One or two embryos were transferred between days 2 and 6. Luteal-phase support was given in all patients.

Control group. From the Monash IVF Australia database of over 10,000 women, we identified a potential cohort of controls who had undergone two cycles within 1 year that had identical FSH dose and stimulation protocols. We ran a 4:1 match using the *genetic* mode algorithm of MatchIt,[52] which runs a variation of propensity matching, to create a cohort that was comparable for age, stimulation type, cycle number, FSH dose and type, year and location, index cycle outcomes, and gap between index and therapeutic cycles. From this cohort, we manually excluded controls with evidence of previous antioxidant use or any therapy known or suspected of affecting oocyte quality. This was assessed through patient records. Out of the remaining cohort, matching was then performed at a 2:1 ratio using the *nearest* mode algorithm of MatchIt on the following confounders: age, stimulation type, FSH dose and type, therapeutic cycle number, gap between cycles, index cycle outcomes (oocyte number, previous cancellations, and pregnancy outcomes), additional FSH therapy, and whether a biopsy was required. This resulted in a final matched cohort

of 106 controls. This provided the optimal 2:1 match for cases to controls as the two closest controls for each case was taken from a cohort that was already comparable to the case group for the matched confounders, with any women on oocyte affecting therapy removed.

Outcomes measured. Our primary outcomes were number of follicles >11 mm, total oocyte number, and metaphase II oocytes. Our secondary outcomes were fertilization rate, utilization rates, and pregnancy outcomes. Utilization rate was defined as the proportion of embryos transferred or frozen out of the total fertilized oocytes. Clinical pregnancies were those with cardiac activity at 6–7 weeks, and biochemical pregnancies were those with a small and transitory hCG rise.

Analysis. For both cases and controls, we measured their outcomes in both the first (index) cycle and second (therapeutic) cycle. This was not used to compare the cases before and after antioxidant use, but was used so that we could identify the median change between the cycles in the case group and compare it to the median change in control group. The median change was used as a measure of the effect of treatment.

We analyzed the results in two ways; first, by direct comparison of cases and controls in the therapeutic cycle, and secondly, by comparison of the median change between cycles. The second analysis was used to help eliminate bias by negating the effect of any underlying differences between the cases and controls, as the groups were matched but not randomized. We also analyzed the cases in subgroups based on age, either <40 or ≥40.

Statistical analysis was conducted using SPSS Statistics version 20 (Chicago, IL, USA). Shapiro–Wilk and Kolmogorov–Smirnov tests were conducted to assess the normality of all data sets. Must data were not normally distributed and were analyzed using Mann–Whitney *U*-tests for continuous variables, with Student's *t*-tests (two-tailed) as appropriate. Categorical variables were analyzed using Fisher's exact tests. Statistical significance was established at $P \leq 0.05$.

Results

As shown in Table 1, cases and matched controls were comparable for age, cycle number, average FSH dose administered, body mass index (BMI), and oocyte number in the first cycle, which was used as a marker of previous cycle success. We did not match on maximum estradiol (E2) level, and there was a significant difference in maximum E2 measured (3,896 vs. 2,836, $P = 0.037$). The median time between cycles was also statistically significant, with a shorter median duration between cycles in the control group (77.5 vs. 108 days, $P = 0.009$).

Once ovarian stimulation had begun, 8 women from the case group and 19 women from the control group had their cycles canceled before oocyte collection in the treatment cycle due to inadequate follicle number (15% vs. 18%, $P = 0.661$).

As seen in Table 2, no difference in follicle number was seen. Absolute oocyte number was higher in the treatment

Table 1. Comparison of demographics and IVF treatments between cases and controls.

	CASES (MEDIAN 95% CI)	CONTROLS (MEDIAN 95% CI)	P-VALUE****
Previous cycle number*	3.0 (3.0–5.0)	3.0 (2.0–4.0)	0.670
Cycle number**	4.0 (4.0–6.0)	4.0 (4.0–5.0)	0.744
FSH dose (IU)	450 (300–450)	450 (400–450)	0.684
BMI	24.0 (23.0–27.0)	24.0 (23.0–25.0)	0.553
Time between cycles (days)	108 (81.0–129)	77.5 (68.0–100)	**0.009**
Age (years)	41.4 (39.6–42.3)	41.4 (40.1–42.0)	0.807
Maximum estradiol (E2)***	3896 (2955–4592)	2836 (2571–3283)	**0.037**
Oocyte number in first cycle	6.0 (4.0–8.0)	6.0 (5.0–7.0)	0.949

Notes: *Previous fresh cycle transfer. **Total cycle number including fresh and frozen transfers. ***Measured during the index cycle. Measured in nanograms per milliliter.

cycle for cases compared to controls (6.00 vs. 4.00, $P = 0.049$). There was no significant difference in follicle number, MII number, or fertilization number.

There were significant differences between the cases and controls when examining the median change between the cycles before and after treatment (see Table 3). Median change in oocyte count (0.00 vs. −1.00, $P = 0.028$) and median change in MII count (0.00 vs. −1.00, $P = 0.034$) were significant. This difference was not the result of a median increase in the case group, but rather a median decrease in the control group.

There was no difference in the proportion of oocytes that developed to maturity in cases versus controls (77% vs. 77%, $P = 0.958$). No difference was seen in fertilization numbers or in the fertilization rate (62% vs. 66%, $P = 0.702$). The utilization rate for cases and controls was equivalent (58% vs. 50%, $P = 0.355$). However, again few conclusions can be drawn from this as it included both cleavage- and blastocyst-stage transfers and freezes, without isolated analysis of each group. There was no difference in overall pregnancy rate (clinical and biochemical) between the cases (13%) and controls (13% vs. 16%, $P = 0.4827$).

When we analyzed the groups according to age, <40 ($n = 21$) and ≥ 40 ($n = 32$), we found a significant difference in mean MII difference between cycles (0.48 vs. −1.55, $P = 0.018$) in the ≥ 40 group. In the ≥ 40 group, we also saw a trend toward significance in absolute mean oocyte number (6.34 vs. 4.36, $P = 0.057$) and mean oocyte difference (−1.63 vs. −0.19, $P = 0.072$). No differences were seen in any primary outcome in the <40 cohort.

There were no differences in any primary or secondary outcome when comparing the melatonin-only ($n = 34$) group to the melatonin and CoQ10 ($n = 19$) group.

Discussion

We did see a statistically significant difference between the cases and controls after antioxidant therapy in absolute oocyte number and median change in both oocyte and MII number. However, from our results we cannot conclude that melatonin

Table 2. Comparison of primary outcomes between cases and controls.

OUTCOME	CONTROL CYCLE MEDIAN (95% CI)	TREATMENT CYCLE MEDIAN (95% CI)	TREATMENT CYCLE MEAN*	IQR***	P-VALUE**
Follicle number					
Cases	7.0 (5.0–9.0)	6.0 (5.0–9.0)	7.47	4.00–10.0	0.138
Controls	6.0 (5.0–7.0)	6.0 (5.0–7.0)	6.04	3.00–8.75	
Oocyte number					
Cases	6.0 (4.0–8.0)	6.0 (5.0–9.0)	7.21	4.00–10.0	**0.049**
Controls	6.0 (5.0–7.0)	4.0 (3.0–6.0)	5.57	2.00–8.75	
MII number					
Cases	5.0 (3.0–6.0)	5.0 (2.0–6.0)	5.42	2.00–8.00	0.114
Controls	5.0 (4.0–6.0)	3.0 (3.0–7.0)	4.32	0.5–7.00	
Fertilization number					
Cases	3.0 (2.0–4.0)	2.0 (2.0–3.0)	3.00	1.00–5.00	0.087
Controls	3.0 (2.0–3.0)	2.0 (1.0–3.0)	2.57	0.00–4.00	

Notes: *Means are provided for reference only. Data were nonparametric and analysis was conducted by Mann–Whitney U-tests. *P-value is for the direct comparison of outcomes in the treatment cycle between the cases and controls. Mann–Whitney U-tests were used. ***Interquartile range (25th–75th centiles).

Table 3. Comparison of median differences between control and therapeutic cycles.

OUTCOME	MEDIAN CHANGE (95% CI)	P-VALUE*
Follicle number		
Cases	−1.0 (−2.0–0.0)	0.926
Controls	−1.0 (−1.0–0.0)	
Oocyte number		
Cases	0.0 (−1.0–2.0)	**0.028**
Controls	−1.0 (−2.0–0.0)	
MII number		
Cases	0.0 (0.0–2.0)	**0.034**
Controls	−1.0 (−1.0–3.0)	
Fertilization number		
Cases	0.0 (−1.0–1.0)	0.105
Controls	−1.0 (−1.0–0.0)	

Notes: *P-value is for the comparison of the Mann–Whitney U-tests used for analysis.

with or without CoQ10 improves oocyte quality or quantity. This is because the significant median differences were the result of a reduction in oocyte and MII number in the second cycle of the control group, rather than an improvement in the case group. Although the lower oocyte and MII number seen in the second cycle of the control group may be the result of age related decline in oocyte number over time, it may also be due to chance. Without any improvement seen in oocyte or MII number in the case group, although we can conclude that the results in the case and control group were significantly different, we cannot necessarily conclude that antioxidant therapy improves clinical outcomes.

How do our results compare to the literature? The most consistent finding within the melatonin studies has been an increase in MII count[26,30,53] or a higher proportion of immature oocytes developing to maturity after melatonin.[26,27,29] This alights with the theoretical basis of antioxidant therapy in improving mitochondrial function and overcoming arrested development. We did see significant differences in oocyte and MII numbers compared to controls, however as discussed, these were not due to improvements in the case group, but rather reductions in the control group. We did not see any change in development to maturity in our study. Increases in fertilisation rates have been seen in only a few studies[37,53,54] and we saw no differences in our results.

Our study has several inherent sources of bias, stemming from the lack of randomization and the retrospective nature of a case control study. It is very difficult to ensure that women from the control group did not purchase either melatonin or CoQ10 without disclosing this to the treating team. However, these are therapies that are specifically inquired about and recorded at the time of oocyte collection, so reducing the risk of bias. As the study was retrospective and the results analyzed

after the women had already been prescribed therapy, a proportion of the cases in the latter part of the study were commenced on melatonin 3 months prior to, rather than on the day of, follicle stimulation. This means that we cannot comment on our results in relation to length of treatment. As follicular development can take 90 days, commencement in advance of an IVF cycle appears logical to ensure maximal effects; however, this is not always practical with cycle timing. The relatively small sample size used in this study may mean that we did not have sufficient power to detect true differences. Additionally, it also increases the likelihood that the significant differences we saw in oocyte number were not true differences.

Ideally, live birth rate would have been our primary outcome. However, given the baseline characteristics of our cohort, with very low expected pregnancy rates, a much larger trial would have been required to investigate this.

We did not directly measure free radical or antioxidant concentrations in the follicular fluid of the women in our study, either before or after treatment. As such, we can only hypothesize about whether the antioxidant administration affected the level of oxidative stress in the follicular fluid. However, other studies have indicated that similar dosages of melatonin have resulted in reductions in oxidative stress.[37] Additionally, we did not measure CoQ10 levels. We expected the women on CoQ10 supplementation to have a CoQ10 deficiency, given the documented relationship between age and CoQ10 levels.[46] However, if the women were not deficient, then the CoQ10 supplementation would have no effect. Bentov et al in 2014[49] showed a reduction in aneuploidy rates but significant change in embryo quality with a 600-mg dosage of CoQ10. This was published after the commencement of our study. As they did not see any significant results with a higher dose of CoQ10, it is not unexpected that we did not see any additional benefit with our 150-mg dosage of CoQ10. Our dose may well be sub therapeutic if used for the purpose of improving oocyte quality, and further research is necessary to determine what dose, if any, could benefit oocyte quality.

Other studies involving melatonin have examined women who have independent risk factors for high levels of oxidative stress, including poor sleep,[26] late maternal age,[30] or polycystic ovarian syndrome.[28] It must be considered whether antioxidants may have a universal effect on improving oocyte quality or quantity when undergoing the IVF process, or whether only a subgroup of women will respond. It was interesting to note that the older cohort (≥40) appeared to perform better than the younger cohort, given that oxidative stress levels are known to increase with age. Although this is essentially observational given the limits in sample size, it may be worth conducting future investigations into women with high levels of oxidative stress, such as smokers, women with very high or low body mass indexes, or further examining age differences. It is important to differentiate between the categories of patients who may respond to the therapy, as the cost of even inexpensive adjuvant therapies can accumulate, and we should limit

arbitrary use in patients who are already faced with high financial burdens when undergoing infertility treatments.

Author Contributions

Conceived and designed the experiments: NP, BV, LB, TO, and MH. Collected the data: NP, SS, KR, and FS. Analyzed the data: MH, NP, KR, SJ, SS, and KS. Wrote the first draft of the manuscript: NP. Contributed to the writing of the manuscript: NP, VK, BV, MH, SJ, and FS. Jointly developed the structure and arguments for the paper. KS, MH, NP, TO, SJ, BV, and LB. Made critical revisions and approved final version: NP, BV, MH, KS, VK, SJ, KR, SS, LB, TO, and FS. All authors have reviewed and approved the final manuscript.

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