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The effects of melatonin on follicular oxidative stress and art outcomes in women with diminished ovarian reserve: a randomized controlled trial

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Abstract

Background To investigate the impact of Melatonin on follicular oxidative stress and assisted reproductive technology (ART) outcomes in women with diminished ovarian reserve (DOR).

Method We put 68 women with DOR who were going through ART into a randomized controlled trial. Starting on the fifth day of their menstrual cycle, we gave them either 3 mg of Melatonin or a placebo every day before stimulating their ovaries. We obtained follicular fluid during oocyte retrieval, assessed it for oxidative stress indicators, and documented ART outcomes.

Results Melatonin administration markedly enhanced the quantity of oocytes retrieved, fertilization rates, and embryo quality. In addition, Melatonin changed markers of oxidative stress, specifically the levels of reduced glutathione (rGSH) and total antioxidant capacity (TAC). The Melatonin group exhibited significantly elevated biochemical pregnancy rates.

Conclusion Melatonin may improve the quality of oocytes and help with reproductive technology in women with low ovarian reserves, possibly by lowering oxidative stress in the follicles.

Keywords Oxidative stress, Follicular fluid, Diminished ovarian reserve, Melatonin

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Introduction

Infertility constitutes not merely a medical issue but also a societal worry, with a rising incidence in both industrialized and developing nations [1]. Ovarian reserve indicates a woman's reproductive capacity and pertains to the quantity and quality of follicles at various developmental stages within the ovary. Patients with DOR exhibit compromised ovarian capacity, an increased likelihood of inadequate response to ovarian stimulation, a reduced number of oocytes recovered, and elevated cycle cancellation rates, significantly diminishing the probability of conception. The ovarian reserve pertains to both the amount and quality of oocytes. The decline in ovarian reserve associated with aging results from two processes: cellular dysfunction due to the accumulation of reactive oxygen species (ROS) and impaired antioxidant defense mechanisms. Oxidative stress denotes an imbalance between reactive oxygen species (ROS) and the antioxidative defense system, resulting in oxidative damage to DNA, proteins, and lipids. The ovarian reserve significantly influences ovarian aging [2]. Oxidative stress levels in the follicular fluid impose a local and direct impact on the milieu for oocyte formation [3]. Follicular fluid (FF) composition mirrors the follicular microenvironment surrounding the oocyte. It is linked to follicle development and oocyte competence [4–6], serving as a valuable tool to investigate the mechanisms underlying various ovarian disorders. Oxidative stress can impact oocyte quality, influencing female reproduction.

Recent investigations have revealed that Melatonin functions as a free radical scavenger and promotes the activity of antioxidant enzymes, thereby safeguarding cells against oxidative stress [7, 8]. Consequently, Melatonin supplementation has the potential to protect oocytes from oxidative stress, which can contribute to unsuccessful reproductive outcomes in women undergoing ART [9]. Intracellular antioxidant systems, including glutathione (GSH) and ascorbic acid, can mitigate the detrimental effects of reactive oxygen species (ROS). Consequently, maintaining a balance between ROS production and their detoxification is crucial for the quality of oocytes. Studies have shown that melatonin (N-acetyl-5-methoxytryptamine) is an effective free radical scavenger and broad-spectrum antioxidant [10]. Melatonin is a widely distributed and functionally diverse molecule that can influence cells' physiology and molecular biology through various mechanisms [11]. In humans, Melatonin is the primary secretory product of the pineal gland and plays a role in multiple physiological manifestations [3]. Despite investigators observing the influence of Melatonin on mammalian reproductive activities decades ago [12], conflicting findings regarding its effects on the reproductive axis persist. Early research shows that Melatonin can change the way the brain and pituitary gland work to control reproduction by activating receptors in the hypothalamic-pituitary-gonadal axis [13].

Nonetheless, additional research has shown that Melatonin does not directly affect GnRH neurons [14]. The surrounding follicular fluid protects and sustains oocytes, acting as a biological 'window' that reflects oocytes' and granulosa cells' metabolic and hormonal activities [15]. In the environment these cells reside in, Human follicular fluid [16] exhibits a significantly elevated concentration of Melatonin, nearly three times greater than that of serum [17]. Both active ovarian uptake [18] from the general circulation and ovarian synthesis [19] appear to sustain these concentrations. Human granulosa cells [12] produce Melatonin, which enters the follicular fluid. Melatonin removes free radicals and controls the transcription of genes for antioxidative enzymes [20]. This presence and activity of melatonin keeps oocytes safe from oxidative stress [16] in follicular fluid and may also directly affect the quality of oocytes.

Researchers are paying much attention to the positive effects of adding melatonin to culture media during animal studies, such as protecting oocytes in vitro [21, 22] and helping embryos grow [23, 24]. Nonetheless, the exact function of endogenous melatonin in human follicular fluid, particularly in terms of its correlation with IVF outcomes for infertile women, has yet to be established. The study's goal was to find out how Melatonin affected follicular oxidative stress and the results of ART in women with low ovarian reserve who were going through ovarian stimulation cycles. The exact function of Melatonin in human follicular fluid and its correlation with IVF results in infertile women with DOR remain ambiguous. This study examines melatonin supplementation's impact on follicular oxidative stress and ART outcomes in women with DOR following ovarian stimulation. We propose that Melatonin will diminish oxidative stress and enhance critical ART outcomes, such as oocyte yield, fertilization rate, embryo quality, and clinical pregnancy rate.

Materials and methods

Study participants

The current study, which was carried out over a 12-month duration beginning in May 2023, enlisted infertile women referred to the Infertility Center at Kowsar Hospital, along with Urmia University of Medical Sciences. The inclusion criteria consisted of first-time ART, normal male factor, normal uterine cavity, and the existence of two of the following three criteria: (1) bilateral AFC summation ≤ 6, (2) AMH ≤ 1, and (3) basal FSH on the third day of the menstrual cycle ≥ 10. These cases are typically eligible for egg donation at our center. This experiment included couples who demanded the use of their oocytes for assisted reproductive technology. We informed the

couples about the double-blind design of the study. The study excluded cases where the couple declined to participate, failed to adhere to the protocol strictly, or showed inadequate responses to gonadotropins in their ovaries. We screened 126 women for this study. Of those, 20 did not meet the selection criteria, and 22 declined to participate, leaving 84 participants. We then randomly divided these participants into two groups. However, we excluded eight patients from the melatonin group and eight patients from the control group due to their unwillingness to continue. This left us with a final sample of 68 participants: 34 in the treatment group and 34 in the placebo group. Figure 1 presents the CONSORT flow diagram of participant progress throughout the study. Capsules containing 3 mg Melatonin (Hakim Pharmaceutical Company, Iran) and The Pharmaceutics Division of Urmia University of Medical Sciences prepared placebos with the same shapes for the study. To design a double-blind, simple randomization, the pharmaceutical company assigned a code number ranging from 101 to 200 to each package containing either the placebo or Melatonin, as determined by a randomization table. The pharmaceutical company distributed the prepared packages, each containing 34 capsules, to the participants, who were organised based on their entry into the project. The patients and clinicians were unaware of which packet contained the placebo or Melatonin. We advised the participants to ingest one capsule orally each night before bedtime, starting on the 3-5th day of the previous menstrual cycle until the oocyte retrieval. We conducted a transvaginal ultrasound for all participants before to supplement initiation and advised them to continue the supplement until ovum pickup.

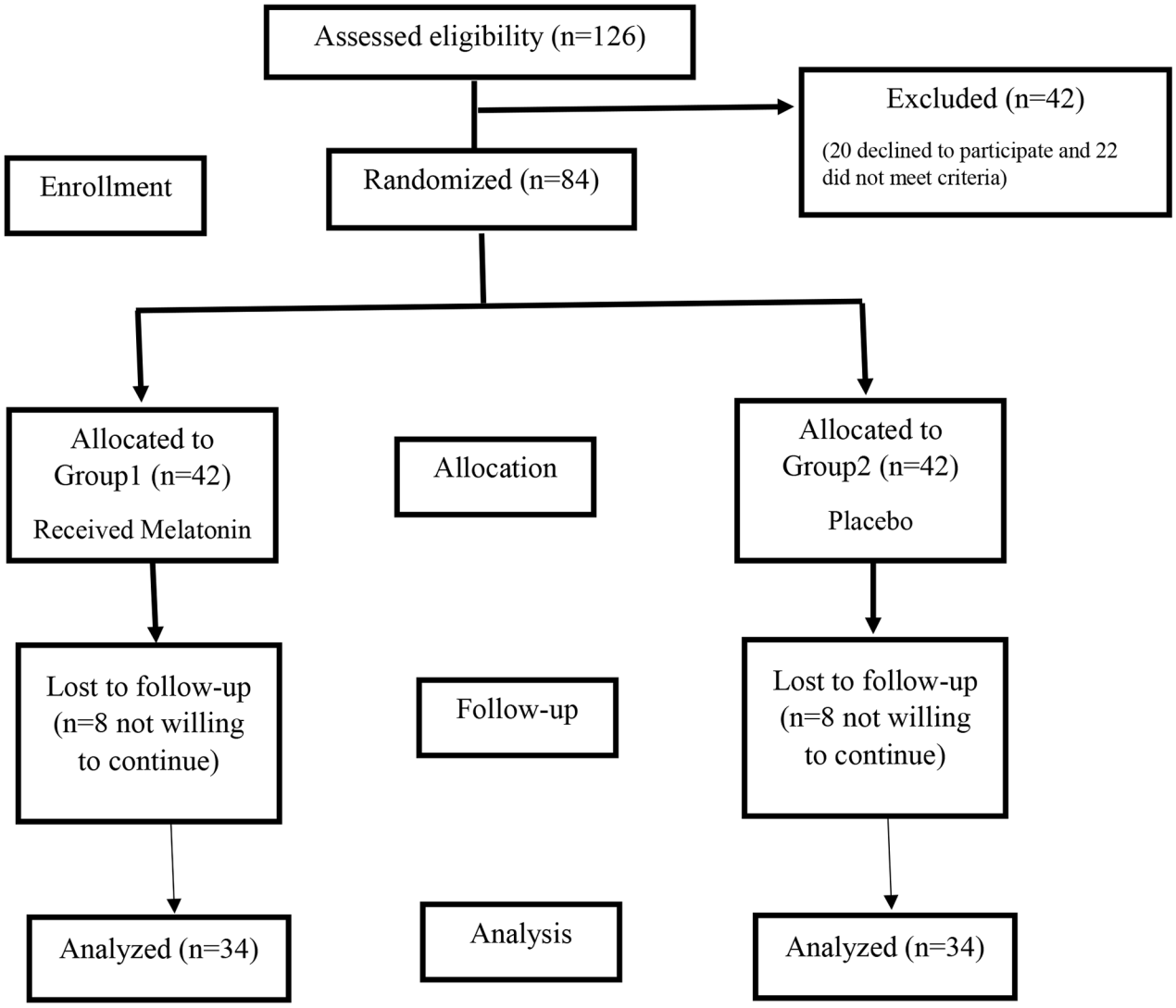

Fig. 1 The study CONSORT flowchart

We employed a long protocol with a GnRH agonist for ovarian stimulation. We provided Leuprolide 1 mg daily from day 20 of the previous cycle until the onset of the subsequent menstrual cycle. We decreased the Leuprolide dosage to 0.5 mg on the second or third day of the new cycle. At the same time, the ovarian response led to a gradual increase in hMG injections from 225 IU to 450 IU. We meticulously observed follicular development using ultrasonography. We injected HCG 5000 IU(PD PREG, Poyesh Darou, Tehran, Iran) to facilitate oocyte maturation once a minimum of two dominant follicles measuring 18 mm or larger emerged. Oocyte retrieval occurred 34 to 36 h post-triggering. We supplemented the luteal phase with 100 mg of intramuscular progesterone (Iran Hormone, Tehran, Iran) daily, starting on the day of oocyte retrieval and continuing for 3 days. We then administered 400 mg of vaginal progesterone (Cyclogest®, Actavis UK Ltd., Barnstaple, UK) once daily. We followed up with the patients and recorded their cycle outcomes. Based on established criteria [4, 5], we categorized the oocytes into three groups: germinal vesicle, metaphase I (MI), and metaphase II (MII). Steer [6] established standard criteria, categorizing the embryos into four grades [1–4], with grade 1 indicating the highest quality. We classified embryos exhibiting less than 20% fragmentation (grade 1 or 2) and more than six blastomeres on day 3 as top-quality embryos. We conducted embryo grading immediately before embryo transfer. All patients underwent transfer of day-3 embryos in a fresh cycle. Double cleavage-stage embryo transfer were performed by using a Wallace embryo transfer catheter with ultrasound guidance. We classified an increase in serum-hCG levels 16 days post-embryo transfer as a biochemical pregnancy. The observation of an embryo exhibiting heart activity at 6–7 weeks gestation confirmed a clinical pregnancy. After collecting the data, we deciphered the pharmaceutical codes from the packages and analyzed the data. We evaluated the case and control groups based on outcomes such as stimulation duration, gonadotropin dosage, serum estradiol levels on the triggering day, quantity and quality of oocytes and embryos, and clinical pregnancies. The Urmia University of Medical Sciences Ethics Committee accepted this study (IR.UMSU.REC.1401.262 and IRCT20170529034209N2).

Follicular fluid collection

We obtained follicular fluid during preovulation and oocyte retrieval. We then centrifuged the Follicular fluid at 2000 rpm for 15 min at 4 °C to exclude insoluble particles and cells. Next, we transferred the supernatant to a 2 ml freezing tube, snap-froze it in liquid nitrogen, and stored it at -80 °C until rGSH, MDA, and TAC analysis.

Immunoassay and oxidative marker measurements

Reduced GSH (Cat# ZB-GSH-48 A), TAC (Cat# ZB-TAC-48 A) and MDA (Cat# ZB-MDA-48 A) were all measured according to the manufacturer's instructions (Zellbio, Biotechnology Company in Lonsee, Germany.

Statistical analysis

We assessed the normality of distribution in continuous data using a normal probability plot, a quantile normal plot, and the Kolmogorov-Smirnov test. We then assessed group differences using an unpaired t-test or a two-sample Wilcoxon rank-sum (Mann-Whitney) test. Additionally, we examined the effects of Melatonin treatment on multiple outcomes, such as the number of oocytes retrieved, the count of MII oocytes, fertilization rate, high-quality embryos, GSH, TAC, MDA, estradiol trigger day, Human menopausal gonadotropin (HMG) dose, and stimulation duration, employing a linear regression model that included age, FSH, AMH, and AFC as explanatory variables. To mitigate non-normality and heteroscedasticity in the residual distributions, we utilized a non-parametric bootstrap method with 2,000 replications to get empirical standard errors and bias-corrected and accelerated (BCa) percentile confidence intervals. Continuous data were presented as mean ± standard deviation (SD) or median (interquartile range). All statistical analyses were performed using R (version 4.0.2), with a significance criterion established at $P<0.05$ or confidence intervals that excluded the null value.

Results

We conducted a statistical analyses to assess the equivalence of baseline features between the Melatonin intervention group and the placebo control group. Our study reveals that 33 women (44.83%) underwent biochemical pregnancy, while 38.2% experienced clinical pregnancy. The study indicated that the *p*-values for all tested variables were above the standard significance threshold of 0.05. This finding indicates that the disparities in baseline characteristics between the treatment and control groups were not statistically significant (Table 1).

The results show that giving Melatonin significantly improves many aspects of reproduction compared to the placebo group. These include the number of oocytes recovered, the fertilization rate, and the embryo quality. Moreover, Melatonin affects oxidative stress indicators, as seen by alterations in rGSH and TAC levels. Nonetheless, many measures, like MDA, exhibited no significant differences (Table 2). The regression analysis demonstrates that Melatonin positively influences many reproductive outcomes, such as the number of oocytes retrieved, fertilization rates, and the production of high-quality embryos while exhibiting a strong negative

Table 1 Baseline demographic and clinical data of patients in the placebo and melatonin groups who completed the study

Variable	Intervention group ($n=35$)	Control group ($n=33$)	*P*-value
	Mean ± SD	Mean ± SD	
Age(y)	34.56 ± 4.69	34.73 ± 5.04	0.882
FSH(IU/L)	14.15 ± 2.35	13.89 ± 1.92	0.619
AMH(ng/mL)	0.79 ± 0.17	0.75 ± 0.21	0.791
AFC(n)	4.24 ± 1.23	3.9 ± 1.13	0.239

Table 2 Comparison of reproductive outcomes in the melatonin and placebo group

Variable	Intervention group ($n=35$)	Control group ($n=33$)	*P*-value
Number of retrieved oocytes			
Mean ± SD	7.38 ± 1.65	3.62 ± 0.89	
Median (IQR)	8.0 (6.0–9.0)	3.0 (3.0–5.0)	< 0.001
Number of MII oocytes			
Mean ± SD	5.91 ± 1.68	2.35 ± 0.59	
Median (IQR)	6.0 (5.0–7.0)	2.0 (2.0–3.0)	< 0.001
Fertilization Rate			
Mean ± SD	65.68 ± 14.45	43.67 ± 12.62	
Median (IQR)	66.5 (57.5–75.5)	41.5 (33.5–51.5)	< 0.001
High quality embryo			
Mean ± SD	43.54 ± 16.21	29.10 ± 11.22	
Median (IQR)	42.5 (30.0–57.5)	30.5 (20.5–35.5)	0.002
rGSH(μmol/L)			
Mean ± SD	4.99 ± 0.96	3.82 ± 1.04	
Median (IQR)	5.03 (4.17–5.63)	3.58 (2.91–4.29)	< 0.001
TAC(U/mL)			
Mean ± SD	0.978 ± 0.06	1.06 ± 0.04	
Median (IQR)	1.01 (0.92–1.03)	1.08 (1.03–1.09)	< 0.001
MDA(nmol/mL)			
Mean ± SD	1.95 ± 0.29	2.01 ± 0.33	
Median (IQR)	1.91 (1.72–2.23)	1.96 (1.69–2.19)	0.556
estradiol trigger day			
Mean ± SD	1867.35 ± 807.41	1236.88 ± 560.97	
Median (IQR)	1856.5 (1100–2470)	1135 (843–1490)	0.002
HMG dose(IU)			
Mean ± SD	3917.21 ± 886.45	3805.53 ± 864.59	
Median (IQR)	3904 (3240–4452)	3681.5 (2965–4359)	0.598
stimulation days			
Mean ± SD	10.17 ± 0.97	11.85 ± 1.31	
Median (IQR)	10.0 (9.3–10.9)	12.0 (11.0–12.0)	< 0.001

correlation with stimulation days and TAC. Furthermore, the Melatonin group significantly increased the incidence of clinical pregnancy. The findings underscore the prospective advantages of Melatonin in reproductive health; nevertheless, certain variables, including MDA, biochemical pregnancy, and HMG dose, did not exhibit significant correlations (Table 3).

Discussion

Oxidative stress levels in follicular fluid directly influence the microenvironment for oocyte development. Research indicates that follicular fluid in patients with DOR correlates with heightened oxidative stress and inflammatory markers. Reduced antioxidant status and increased inflammatory levels in follicular fluid indicate patients with DOR [25]. Huang et al. did a study where they looked at oxidative stress and inflammatory markers in the follicular fluid of women with DOR and normal ovarian reserve (NOR). In addition, they examined how these markers affected embryo quality [25]. Higher levels of oxidative stress and inflammation in the follicular fluid of women with DOR may link to the pathogenesis of DOR [25].

The oxidant-antioxidant state of follicular fluid and its influence on oocytes and IVF results have received much study in recent years [26]. Oxidative stress is associated with damage to oocytes and embryos, which may result in reduced fertilization rates and compromised embryo

Table 3 Regression analysis reflecting the effect of melatonin on post-intervention values after adjustment for their baseline values

Variable	Coefficient (β)	Bootstrap Se	*P* value	95% CI
Number of oocytes retrieved	3.69	0.36	< 0.001	2.99–4.39
Number of MII oocytes	3.46	0.34	0.002	2.77–4.14
Fertilization Rate	20.73	5.35	< 0.001	13.14–28.33
High quality embryo	17.77	3.69	0.03	10.53–25.01
rGSH (μmol/L)	1.15	4.45	< 0.001	0.65–1.67
TAC (U/mL)	-0.09	-6.9	< 0.001	-0.12 – -0.06
MDA (nmol/mL)	-0.013	0.075	0.854	-0.16–0.13
Estradiol triger day	723.88	172.96	0.003	384.87–1062.89
HMG dose(IU)	344.35	230.86	0.136	-108.12–796.82
stimulation days	-179	0.324	< 0.001	-2.43 – -1.16

quality [26]. All cells synthesize Melatonin, an indolamine that exhibits significant antioxidant properties and can directly scavenge free radicals without receptors, acting as a reactive oxygen species (ROS) scavenger [27]. Research indicates that MT's antioxidant capabilities affect the HPG axis [16–18]. MT can mitigate oxidative damage in the follicles, enhance progesterone synthesis during the luteal phase and facilitate oocyte maturation [19]. Prior research has demonstrated that Melatonin facilitates oocyte maturation, fertilization, and embryo development by safeguarding oocytes and associated follicular cells from oxidative injury. In this setting, Melatonin neutralizes free radicals and diminishes oxidative stress in ovarian follicles, consequently safeguarding oocytes and granulosa cells [18, 28].

Moreover, the markedly elevated expression of poly ADP-ribose (PAR) and nuclear translocation of apoptosis-inducing factor (AIF) in the cumulus granulosa cells of patients with DOR suggests a potential association between PARP1-dependent cell death and DOR [29]. Melatonin therapy efficiently suppresses poly ADP-ribosylation (PARylation) and prevents AIF translocation to the nucleus, thereby diminishing the risk of apoptosis in granulosa cells [29]. The quality of oocytes is a critical determinant of IVF success [30]. Oocytes of inferior quality exhibit a decreased probability of fertilization and subsequent development into viable embryos [30]. Melatonin may enhance oocyte quality by augmenting mitochondrial energy production and diminishing DNA damage [31, 32]. We conducted a double-blind, placebo-controlled clinical study on women with DOR to evaluate the potential benefits of Melatonin compared to placebo on oxidative stress levels in follicular fluid and ART outcomes. We included women who satisfied two of the following three criteria: antral follicle count (AFC) ≤ 5, anti-Müllerian hormone (AMH) ≤ 1 ng/mL, and follicle-stimulating hormone (FSH) > 10 IU/L. These rigorous criteria guaranteed the selection of women with markedly reduced ovarian reserves. Our center's standard protocols advocate oocyte donation for patients with severely DOR. Nonetheless, if patients insisted on using their oocytes for their initial ART cycle, we included them in this experiment following comprehensive explanations and the acquisition of written informed consent.

The results of our investigation indicated that Melatonin intake elevated the levels of reduced glutathione (rGSH) and total antioxidant capacity (TAC), signifying a decrease in oxidative stress, whereas malondialdehyde (MDA) levels remained relatively unchanged. The current study demonstrated that oral Melatonin intake in women with DOR enhances IVF results by decreasing follicular oxidative stress. These findings corroborate prior research indicating the beneficial effects of Melatonin on IVF outcomes in women with DOR [33]. Our research has shown that Melatonin intake in women with DOR results in elevated serum estradiol levels on the day of ovulation induction [33]. The antioxidant properties of Melatonin may be responsible for the elevation of blood estradiol levels in the Melatonin group. Oxidative stress may impair granulosa cells and diminish estrogen synthesis [34]. Melatonin enhances granulosa cell activity and elevates estradiol production by mitigating oxidative stress [35]. Comparable investigations have likewise indicated elevated mean estrogen levels following Melatonin intake.

Our study shows that giving melatonin leads to significant improvements in the results of IVF, including the number of retrieved oocytes, mature (metaphase II) oocytes, the rate of fertilization, and the quality of the embryo compared to the placebo group. This is because Melatonin lowers follicular oxidative stress. These findings align with other research demonstrating the beneficial impact of Melatonin on IVF outcomes in women with DOR [33]. Jahromi et al.'s trial showed a higher quantity of MII oocytes in the Melatonin group compared to the placebo group; however, this difference did not reach statistical significance, unlike our findings [33]. Fernando et al.'s study, in contrast to Jahromi et al.'s and our research, found no significant differences in the total number of oocytes, the number of fertilized oocytes, or the quantity and quality of embryos among the groups [36]. The regression analysis of our study revealed a

significant increase in biochemical pregnancy rates in the Melatonin group ($P=0.026$), However, the two groups showed no significant difference in clinical pregnancy rates ($P=0.414$).

This investigation noted a beneficial impact of Melatonin on biochemical pregnancy but no significant effect on clinical pregnancy. A recent study added Melatonin to the in vitro maturation (IVM) culture medium (10 μmol/L) for women with polycystic ovarian syndrome (PCOS). This led to a higher clinical pregnancy rate, though it was not statistically significant [37]. Despite variations in the total number of treatment days and Melatonin dosage throughout the three investigations, the adequate daily amount of Melatonin remains ambiguous. Data suggests that Melatonin is generally safe for oral consumption at varying doses and durations, but adverse effects such as dizziness, headache, nausea, and drowsiness have been documented [38]. Unlike laboratory or animal studies, ethical constraints in human studies prevent researchers from developing projects with several escalating doses. A controlled randomized study showed that giving Melatonin to women with DOR significantly increases the fertilization rate, number of retrieved oocytes and high-quality embryos [33]. A study indicated that Melatonin lowers oxidative stress levels in women's follicular fluids with DOR.

Nonetheless, there are several significant distinctions between our work and prior research. Our study provided Melatonin for the 3rd to 5th day of the previous menstrual cycle until the oocyte retrieval. Secondly, our study concurrently assessed various outcomes, encompassing follicular fluid oxidative stress indicators and IVF results. We observed no difference in clinical pregnancy rates between the groups. This finding is consistent with the findings of the two previously mentioned studies. This study has multiple drawbacks. The sample size was limited. Secondly, our research only focused on women with DOR. The efficacy of Melatonin in women without DOR is uncertain. Future research may reveal that Melatonin supplementation is advantageous in cases of normal ovarian function in enhancing ART outcomes.

Furthermore, researchers could design their investigations to include different dosages and simultaneously measure the Melatonin levels in the follicular fluid of oocytes of varying quality to determine the most effective Melatonin dose. Alternatively, researchers could incorporate Melatonin into a study culture medium to assess the attainment of enhanced ART outcomes. These studies should evaluate the long-term impact of melatonin on IVF outcomes in women with DOR, as well as the appropriate dosage of melatonin.

Additionally, further research is required to explore the mechanisms by which Melatonin enhances IVF outcomes. It is essential to assess the efficacy of Melatonin in women without DOR, and in other groups, including those with endometriosis or polycystic ovary syndrome. Additional research with bigger sample sizes and extended follow-up is required to validate these findings and examine the effects of Melatonin on long-term ART outcomes, including miscarriage and live birth rates.

Conclusion

This study presents persuasive evidence that Melatonin is a potential supplementary medication for enhancing IVF outcomes in women with DOR. Further investigation is required to overcome this study's limitations and examine the prospective therapeutic applications of Melatonin.

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Author contributions
H.G.B and S.S developed the concept and designed the study. T.B.L and S.Scollected the samples. M.G.B and H.G.B performed the measurement. R.B, and H.H analysed the data and interpreted the results. S.S and M.G.B draftedthe manuscript. All authors critically reviewed the manuscript and approvedthe final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committees of Urmia University of Medical Sciences (IR.UMSU.REC.1401.262 and IRCT20170529034209N2). Before the study, all subjects signed informed consent. This was a prospective study of patients undergoing fertility treatment at our IVF center.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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