Reproductive Biology and Endocrinology

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# Melatonin supplementation attenuates cuproptosis and ferroptosis in aging cumulus and granulosa cells: potential for improving IVF outcomes in advanced maternal age

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## Abstract

**Background** Advanced maternal age is associated with decreased oocyte quantity and quality and in vitro fertilization (IVF) success rates. This study aimed to investigate whether melatonin supplementation can improve IVF outcomes in women of advanced maternal age by modulating cuproptosis and ferroptosis.

**Methods** This prospective cohort study included 161 women aged 35–45 years undergoing IVF-frozen embryo transfer cycles. Participants were assigned to either melatonin ($n=86$, 2 mg daily for ≥ 8 weeks) or control ($n=75$) groups. Cumulus cells were analyzed for cuproptosis and ferroptosis-related gene expression. Additional experiments were conducted on the HGL5 human granulosa cell line to assess mitochondrial function and metabolic reprogramming.

**Results** Melatonin supplementation significantly improved IVF outcomes in women aged ≥ 38 years, increasing clinical pregnancy rates (46.0% vs. 20.3%, $P<0.01$), ongoing pregnancy rates (36.5% vs. 15.3%, $P<0.01$), and live birth rates (33.3% vs. 15.3%, $P<0.05$). In cumulus cells from patients, gene expression analysis revealed that melatonin modulated cuproptosis and ferroptosis-related genes, including ATP7B and GPX4, with more pronounced effects in the ≥ 38 years group. This suggests melatonin enhances cellular resilience against oxidative stress and metal-induced toxicity in the ovarian microenvironment. In vitro studies using HGL5 cells showed melatonin reduced oxidative stress markers, improved mitochondrial function, restored expression of glycolysis and TCA cycle-related genes and modulated cuproptosis and ferroptosis-related gene expression. These findings provide mechanistic insight into melatonin's protective effects against regulated cell death in ovarian cells, potentially explaining the improved IVF outcomes observed.

**Conclusions** Melatonin supplementation significantly improved IVF outcomes in women of advanced maternal age, particularly those ≥ 38 years old, likely by modulating cuproptosis and ferroptosis and enhancing mitochondrial function in cumulus and granulosa cells. These results suggest that melatonin could be a promising adjuvant therapy for improving IVF success rates in older women.

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**Keywords** Antioxidant therapy, Reproductive aging, Oocyte quality, Oxidative stress, Assisted reproduction, Mitochondrial function

## Introduction

Advancing maternal age is a significant challenge in reproductive medicine, characterized by a progressive decline in oocyte quantity and quality and diminished fertility potential [1–3]. As women age, their ovarian reserve undergoes a significant reduction, with the remaining oocytes exhibiting increased susceptibility to oxidative stress and mitochondrial dysfunction [4–6]. This age-related decline in oocyte quantity and quality leads to reduced fertilization rates, impaired embryo development, and suboptimal pregnancy outcomes in assisted reproductive technologies (ART), particularly in vitro fertilization (IVF) [7, 8].

The mechanisms underlying ovarian aging and its impact on oocyte quality are complex and multifaceted. Recent advances in cell death research have unveiled novel pathways that may play crucial roles in oocyte and follicular cell health. Among these, cuproptosis and ferroptosis have garnered significant attention due to their potential involvement in age-related cellular dysfunction and death [9, 10]. Cuproptosis, a recently discovered form of programmed cell death, is triggered by the accumulation of copper ions and involves the binding of copper to lipoylated proteins in the tricarboxylic acid (TCA) cycle, leading to protein aggregation and cellular demise [11–13]. Ferroptosis, an iron-dependent form of regulated cell death, is characterized by the accumulation of lipid peroxides and is primarily regulated by the glutathione peroxidase 4 (GPX4) enzyme [14–17].

Given the significant impact of maternal age on reproductive outcomes and the emerging importance of these novel cell death pathways, strategies to enhance oocyte quantity and quality in women of advanced maternal age are of paramount importance in reproductive medicine. Melatonin, a hormone primarily secreted by the pineal gland, has emerged as a promising therapeutic agent in ART [18–20]. Known for its role in regulating circadian rhythms, melatonin also exhibits potent antioxidant properties and influences various physiological processes, including reproduction [21]. In the context of ovarian aging, melatonin has been shown to protect oocytes and surrounding follicular cells from oxidative damage, enhance mitochondrial function, and improve overall oocyte quality [22, 23]. These beneficial effects position melatonin as a potential intervention to address the challenges associated with advanced maternal age in reproductive medicine [24].

However, the interplay between melatonin and these novel cell death pathways in the context of ovarian aging remains largely unexplored. Given melatonin's well-established antioxidant properties and its ability to modulate mitochondrial function [22, 25], we hypothesize that melatonin may influence cuproptosis and ferroptosis in ovarian cells, potentially mitigating the effects of aging on oocyte quality and improving reproductive outcomes. The main objectives of this study are to investigate the effects of melatonin supplementation on IVF outcomes in women of advanced maternal age, to examine the impact of melatonin on cuproptosis and ferroptosis-related gene expression in human cumulus cells and HGL5 cells, and to assess the effects of melatonin on mitochondrial function, oxidative stress, and energy metabolism in HGL5 cells. This study uniquely explores the effects of melatonin on newly discovered cell death pathways, cuproptosis and ferroptosis, in the context of ovarian aging, providing novel insights into potential mechanisms for improving oocyte quality in women of advanced maternal age undergoing ART.

## Materials and methods

### Ethics statement

Ethical approval for this study was obtained from the Institutional Review Board of Kaohsiung Veterans General Hospital (Approval ID: KSVGH21-CT1-43). The study conformed to the ethical principles set forth in the Declaration of Helsinki, ensuring the protection of participants' welfare, rights, and privacy. The review process included a thorough assessment of the study protocol, covering aspects such as participant recruitment, informed consent procedures, data management, and confidentiality safeguards. This comprehensive ethical review underscores our commitment to maintaining the highest standards of research integrity and participant protection throughout the study. All participants provided written informed consent. This study was registered on ClinicalTrials.gov (identifier: NCT06546774).

### Participants and inclusion/exclusion criteria

The study recruited infertile women undergoing frozen embryo transfer (FET) cycles at our reproductive center. The inclusion criteria targeted patients between 35 and 45 years of age with a body mass index (BMI) of 18 to 35 kg/m$^2$. Exclusion criteria were comprehensive, excluding patients with a history of oophorectomy, primary ovarian insufficiency, congenital

uterine anomalies, or severe intrauterine adhesions. Additionally, patients undergoing preimplantation genetic testing for aneuploidy (PGT-A), recipients of donated oocytes, cancer patients, and those who had used hormone treatments or supplements in the past three months were also excluded. A total of 161 women were included and assigned to either the melatonin or control groups. Participants in the melatonin group received a daily dose of 2 mg of melatonin (Somn Well XR Tablet® by Biofrontier Inc., Asia, Taiwan) for at least eight weeks before commencing their IVF cycles. The analysis was conducted separately for all participants aged ≥ 35 years and for a subset of older women aged ≥ 38 years to evaluate the effects of melatonin supplementation.

#### Protocols for controlled ovarian stimulation and frozen embryo transfer

In this study, all participants underwent a GnRH antagonist protocol for controlled ovarian stimulation. Initial evaluations included hormone screening and transvaginal ultrasound to assess antral follicle counts. Ovarian stimulation commenced within 5 days of the menstrual cycle using a combination of recombinant follicle-stimulating hormone and recombinant luteinizing hormone (Pergoveris, Merck Serono SA, Aubonne, Switzerland). Patient response was continuously monitored with serial transvaginal ultrasounds and hormone level assessments. Dosage of the stimulants was adjusted based on the ovarian response.

Daily GnRH antagonist injections (Cetrotide 0.25 mg, Pierre Fabre Medicament Production, Aquitaine Pharm International, Idron, France) were started when the leading follicle reached 12–14 mm in diameter and continued until the final oocyte maturation. A dual-trigger approach was employed, consisting of combined recombinant hCG (Ovidrel 250 μg, Merck Serono S.p.A., Modugno, Italy) and a GnRH agonist (Lupro 2 mg, Nang Kuang Pharmaceutical Co., Ltd., Tainan, Taiwan), when at least one follicle reached a mean diameter of 18 mm. Transvaginal ultrasound-guided oocyte retrieval was performed 36 h after trigger administration. Fertilization was carried out using either conventional IVF or intracytoplasmic sperm injection (ICSI), based on the results of semen analysis or previous fertilization outcomes.

Embryo quality on Day 3 was assessed according to standards established by the Istanbul consensus workshop, evaluating the degree of fragmentation, uniformity of blastomeres, and the presence of multinucleation. Embryos were categorized into Grades 1 to 3. In this study, top-quality Day 3 embryos were those with 6 to 10 cells and classified as Grade 1. This grade was characterized by less than 10% fragmentation, consistent blastomere morphology, and no evidence of multinucleation. For Day 5 embryos, quality was assessed using the Gardner and Schoolcraft scoring system, which includes evaluation of expansion degree (grades 1–6), inner cell mass morphology (grades A-C), and trophectoderm morphology (grades A-C). Top-quality Day 5 embryos were classified as Grade 3AA or better. All embryos were cryopreserved using the vitrification technique under a freeze-all strategy for subsequent FET.

Endometrial preparation was conducted using hormone replacement therapy (HRT). The process began on day 2 or day 3 of the menstrual cycle with daily oral estradiol doses of 6–8 mg (Ediol, Synmosa Biopharma Corporation, Hsinchu County, Taiwan) in combination with estradiol gel (Oestrogel gel, Besins, Drogenbos, Belgium). After 14 days of estrogen therapy, endometrial thickness was measured using a 2-dimensional vaginal ultrasound (Voluson E8, GE Healthcare, Chicago, USA). Once the endometrial thickness reached at least 7 mm, luteal phase support was initiated. This included daily intravaginal administration of 90 mg progesterone gel (Crinone 8% gel, Merck Serono, Hertfordshire, UK), daily oral administration of 30 mg dydrogesterone (Duphaston, Abbott, Olst, the Netherlands), and weekly intramuscular injections of 125 mg progesterone (Progeston Depot, Tafong Pharmaceutical Co., Ltd., Changhua City, Taiwan). For embryo transfer, cleavage-stage embryos were thawed and transferred on the fourth day following progesterone initiation, whereas blastocysts were thawed and transferred on the sixth day. The embryo transfer procedure was meticulously conducted under transabdominal ultrasound guidance to ensure precise placement. Upon confirmation of pregnancy, progesterone supplementation was maintained until the gestational period reached 10–12 weeks.

#### Outcomes measure for IVF-FET cycles

The primary endpoint of this study was the live birth rate, defined as the birth of a viable infant after 24 weeks of gestation. Secondary endpoints included clinical pregnancy rate, ongoing pregnancy rate, and miscarriage rate. Clinical pregnancy was identified through the presence of a fetal heartbeat detected via transvaginal sonography at 6–7 weeks of gestation. Ongoing pregnancy was characterized by the continuation of the pregnancy beyond 12 weeks of gestation. Miscarriage was defined as the loss of a pregnancy following the detection of fetal cardiac activity but occurring before 24 weeks of gestation.

#### Human cumulus cell collection

Following oocyte retrieval, the cumulus-oocyte complexes were collected and washed before being transferred into IVF medium under paraffin oil for further processing. To isolate the cumulus cells, they were initially treated with 40 IU/mL hyaluronidase (SynVitro™ Hyadase, Origo, Knardrupvej, Denmark) for 3 min, followed by thorough washing with phosphate-buffered saline (PBS). Subsequently, the cumulus cells underwent mechanical dissociation and were washed again. The isolated cumulus cells were then resuspended in Histopaque 1077 (Sigma-Aldrich, Waltham, MA, USA) and enriched with a culture medium containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 5 mg/L insulin, 5 mg/L transferrin, 5 µg/L sodium selenite (ITS, Sigma), and 1.25 µM androstenedione (4-androstene-3, 17-dione, Sigma). The resuspended cumulus cells were plated into a 4-well plate at a concentration of $2\times10^4$ viable cells per well. They were then incubated at 37.5 °C in a humidified environment with 5% $CO_2$ for up to 24 h to prepare for subsequent experiments.

#### Protocol for cell culture and treatment

The human ovarian granulosa cell line (HGL5) was obtained from Applied Biological Materials Inc. These cells were cultured under standard conditions in a humidified incubator, maintaining a temperature of 37 °C and 5% $CO_2$. The culture medium consisted of 10% fetal bovine serum, 1% penicillin/streptomycin, 2% Ultroser G (Pall Corp.), and 1% ITS Plus (ZenBio) to support optimal cell growth. The HGL5 cell line was utilized as a model to study cellular senescence, which was induced through serial passaging. In this research, cells that had undergone passages 59 and 113 were deemed senescent. To induce oxidative stress, HGL5 cells were treated with hydrogen peroxide ($H_2O_2$). To assess the potential protective effects of melatonin, cells were pretreated with 25 µM melatonin for 20 h before exposing them to 0.8 mM $H_2O_2$ for 4 h. The objective was to investigate how melatonin alleviates oxidative stress-induced damage in HGL5 cells by comparing the effects observed in melatonin-treated versus untreated cells.

#### Mitochondrial function measurement

Mitochondrial function was assessed using established assays as described previously [20]. Cells from each experimental condition were collected and subjected to a series of staining procedures with specific fluorescent probes designed to assess distinct aspects of mitochondrial function. The following probes were used: CellROX (5 µM) to quantify oxidative stress, DCFDA (5 µM) for general reactive oxygen species (ROS) detection, MitoTracker Green (10 nM) to measure mitochondrial mass, tetramethylrhodamine methyl ester (TMRM; 200 nM) to evaluate mitochondrial membrane potential, and MitoSOX (5 µM) to detect mitochondrial superoxide levels (all probes sourced from Molecular Probes). Staining was performed at 37 °C in a controlled incubator to maintain optimal conditions for probe performance. After staining, cells were thoroughly washed with PBS to eliminate any unbound fluorescent dye. The washed cell pellets were then subjected to centrifugation to concentrate the cells, which were subsequently resuspended in PBS for further analysis. Flow cytometry was employed to quantify the fluorescence intensity of the various probes, providing precise measurements of mitochondrial function and integrity.

#### Oxygen consumption rate measurement

Oxygen consumption rate (OCR) was measured as outlined in a previous study [26]. This analysis was conducted with an extracellular flux analyzer (Agilent Technologies, Santa Clara, CA, USA) utilizing the Seahorse XF HS Mini platform. This platform is designed to provide precise measurements of cellular respiration by monitoring OCR in real-time. For the assays, cells were cultured in specialized assay plates at a density of approximately 2,000 cells per well. During the analysis, OCR was continuously recorded to track dynamic changes in cellular respiration over time. To assess different facets of mitochondrial function, a sequential treatment approach was utilized. Initially, oligomycin was administered to inhibit ATP synthase, thereby measuring the basal respiration and ATP-linked respiration. Following this, FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) was introduced to uncouple oxidative phosphorylation and determine the maximal respiratory capacity of the mitochondria. Finally, antimycin A and rotenone were applied to block the electron transport chain, thereby quantifying the non-mitochondrial respiration and assessing the residual respiration due to other cellular activities. This multi-step approach provided a detailed profile of mitochondrial function, including basal respiration, maximal respiratory capacity, and non-mitochondrial respiration, contributing to a comprehensive understanding of cellular energy metabolism.

#### Western blotting

Western blotting was performed as previously described [27]. The analysis utilized primary antibodies specific for Total OXPHOS (Abcam, catalog number ab110411), NRF2 (Cell Signaling Technology, catalog number 33649), KEAP1 (Proteintech, catalog number 10503-2-AP), and alpha-tubulin (GeneTex,

catalog number GTX628802. The Western blot procedure involved protein extraction, SDS-PAGE separation, and transfer to a nitrocellulose membrane. Following the transfer, the membrane was incubated with the aforementioned primary antibodies, and detection was achieved using appropriate secondary antibodies and chemiluminescent substrates. This methodology provided insights into the expression levels and interactions of the targeted proteins, contributing to a deeper understanding of their roles in cellular processes.

#### RNA extraction and real-time polymerase chain reaction

RNA was extracted using REzol (Protech Technology), a reagent known for its efficacy in isolating high-quality RNA from diverse sample types. Following extraction, mRNA expression levels were quantified using SYBR Green-based quantitative real-time PCR (qRT-PCR) performed on the StepOne system (Applied Biosystems), which is recognized for its precision in gene expression analysis. For accurate normalization of the qRT-PCR data, RNU6-1 was used as the reference gene. RNU6-1 is a small nuclear RNA commonly used as an internal control due to its stable expression across different sample conditions, which ensures consistency and reliability in the measurement of target gene expression. Detailed information regarding the primer sequences utilized for the qRT-PCR assays is provided in Supplementary Table S1.

#### Statistical analysis

For the analysis of patient data, the Kolmogorov–Smirnov test was employed to assess the normality of continuous variables. Quantitative data were compared using independent t-tests, whereas categorical variables were analyzed with either the Chi-square test or Fisher's exact test, as appropriate. All statistical analyses were performed using SPSS version 20.0 (Statistical Package for the Social Sciences, Chicago, IL, USA), with a significance threshold set at $P<0.05$. In the experimental study, data were derived from a minimum of three independent replicates, and results are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Two-way ANOVA, followed by Tukey's post hoc test, was used to determine significant differences between group means, with statistical significance set at $P<0.05$.

## Results

#### Melatonin improves IVF-FET outcomes

Table 1 compares baseline characteristics and IVF-FET outcomes for patients aged ≥ 35 and ≥ 38 years with or without melatonin supplementation. Baseline characteristics were comparable between groups. The melatonin group showed significantly higher numbers of retrieved oocytes, metaphase II oocytes, fertilized oocytes, and top-quality embryos across both age categories. For patients ≥ 38 years, melatonin significantly improved clinical pregnancy rates (46.0% vs. 20.3%, $P<0.01$), ongoing pregnancy rates (36.5% vs. 15.3%,

**Table 1** Baseline and cycle characteristics of patients aged ≥ 35 years or ≥ 38 years undergoing IVF-FET cycles with or without melatonin supplementation

| | Age ≧ 35 years | | | Age ≧ 38 years | | |
|---|---|---|---|---|---|---|
| **Parameters** | **Melatonin (*n* = 86)** | **Control (*n* = 75)** | ***p* value** | **Melatonin (*n* = 63)** | **Control (*n* = 59)** | ***p* value** |
| Age (years) | 39.8 ± 2.8 | 40.1 ± 3.0 | 0.581 | 41.2 ± 1.9 | 41.2 ± 2.3 | 0.954 |
| Body mass index (kg/m$^2$) | 23.1 ± 4.2 | 22.9 ± 3.8 | 0.781 | 23.1 ± 4.4 | 23.2 ± 4.0 | 0.959 |
| Infertility duration (years) | 4.1 ± 2.7 | 4.1 ± 3.2 | 0.972 | 4.3 ± 2.7 | 4.4 ± 3.3 | 0.800 |
| Types of infertility (%) | | | 0.129 | | | 0.049* |
| Primary infertility | 34.9%(30/86) | 46.7%(35/75) | | 28.6%(18/63) | 45.8%(27/59) | |
| Secondary infertility | 65.1%(56/86) | 53.3%(40/75) | | 71.4%(45/63) | 54.2%(32/59) | |
| FSH (mIU/mL) | 6.5 ± 4.1 | 6.4 ± 4.1 | 0.829 | 6.7 ± 4.2 | 6.3 ± 4.0 | 0.657 |
| Anti-Müllerian hormone (ng/ml) | 1.70 ± 1.06 | 1.61 ± 1.17 | 0.626 | 1.57 ± 1.01 | 1.34 ± 0.95 | 0.214 |
| Number of oocytes retrieved (n) | 8.8 ± 5.3 | 7.1 ± 4.1 | 0.022* | 8.2 ± 4.6 | 6.1 ± 3.3 | 0.005** |
| Number of metaphase II oocytes (n) | 7.0 ± 4.8 | 5.5 ± 3.2 | 0.025* | 6.4 ± 4.1 | 4.7 ± 2.6 | 0.010** |
| Maturation rate (%) | 76.8 ± 21.6% | 79.9 ± 18.5% | 0.337 | 76.8 ± 23.3% | 80.6 ± 21.8% | 0.328 |
| Number of oocytes fertilized (n) | 5.8 ± 4.0 | 4.5 ± 2.9 | 0.021* | 5.3 ± 3.4 | 3.8 ± 2.3 | 0.005** |
| Fertilization rate (%) | 84.0 ± 18.0% | 83.3 ± 20.8% | 0.823 | 83.7 ± 18.6% | 81.9 ± 21.7% | 0.618 |
| Number of D3 embryos (n) | 5.5 ± 3.9 | 4.2 ± 2.8 | 0.020* | 5.0 ± 3.3 | 3.5 ± 2.2 | 0.005** |
| Number of top-quality D3 embryos (n) | 2.3 ± 1.9 | 1.7 ± 1.8 | 0.034* | 2.3 ± 1.8 | 1.4 ± 1.3 | 0.003** |

Data are presented as the mean ± standard deviation and % (n)

IVF, in vitro fertilization; FET, frozen embryo transfer; FSH, follicular stimulating hormone; D, day. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

$P<0.01$), and live birth rates (33.3% vs. 15.3%, $P<0.05$) (Fig. 1B). In the ≥35 years category, only clinical pregnancy rates were significantly higher with melatonin (43.0% vs. 26.7%, $P<0.05$) (Fig. 1A).

### Differential expression of cuproptosis and ferroptosis-related genes in aging human cumulus cells following melatonin supplementation

We examined melatonin's effects on cuproptosis and ferroptosis-related gene expression in cumulus cells from women aged ≥35 and ≥38 years. To evaluate the efficacy of melatonin across these age groups, cumulus cells were isolated from patients and subjected to gene expression analysis for cuproptosis-related genes (ATP7B, SLC31A1, FDX1, DLD, DLAT) and ferroptosis-related genes (TFRC, GPX4, NCOA4, SLC3A2, SLC7A11). In the ≥35 years group, melatonin significantly changed expression of SLC31A1 and FDX1 (Fig. 2A). In the ≥38 years group, ATP7B, SLC31A1, FDX1, and DLD were significantly altered. For ferroptosis-related genes, TFRC and SLC3A2 changed significantly in the ≥35 years group, while TFRC, GPX4, NCOA4, and SLC3A2 changed in the ≥38 years group (Fig. 2B).

### Melatonin improves oxidative stress-induced mitochondrial dysfunction and metabolic reprogramming in aging HGL5 cells

We investigated melatonin's effects on oxidative stress and mitochondrial function in HGL5 cells using fluorescent dyes (CellROX, DCFH-DA, and MitoSOX). As shown in Fig. 3, melatonin significantly reduced oxidative stress markers across different cell passages, with a more pronounced effect in older cells (P113 vs. P59). This suggests that HGL5 cells with a greater number of passages are more responsive to melatonin's antioxidative activity. We also evaluated melatonin's impact on mitochondrial membrane potential using TMRM. In $H_2O_2$-exposed cells, melatonin pretreatment markedly restored the compromised mitochondrial membrane potential, as indicated by TMRM fluorescence. These findings suggest that melatonin not only lowers mitochondrial ROS levels but also enhances mitochondrial function by stabilizing the membrane potential. Overall, melatonin demonstrates a protective effect against oxidative stress by improving mitochondrial health and function in aging HGL5 cells.

To explore melatonin's effects on metabolic pathways in oxidatively stressed HGL5 cells, we analyzed

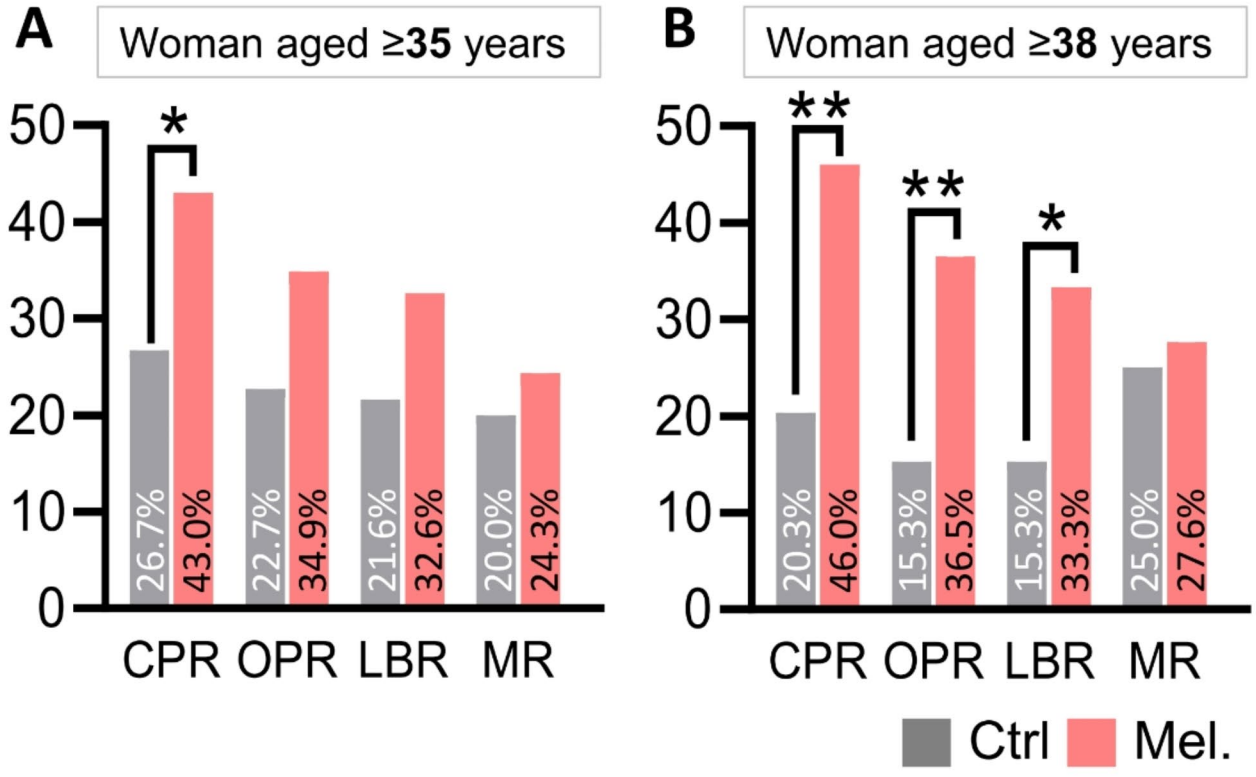


**Fig. 1** Comparison of clinical pregnancy rates, ongoing pregnancy rates, and live birth rates between the melatonin and control groups. Data are presented for women aged ≥35 years (**A**) and women aged ≥38 years (**B**) undergoing IVF-FET cycles. Data were assessed using Chi-square test. Statistical significance is indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$. CPR, clinical pregnancy rate; OPR, ongoing pregnancy rate; LBR, live birth rate; MR, miscarriage rate

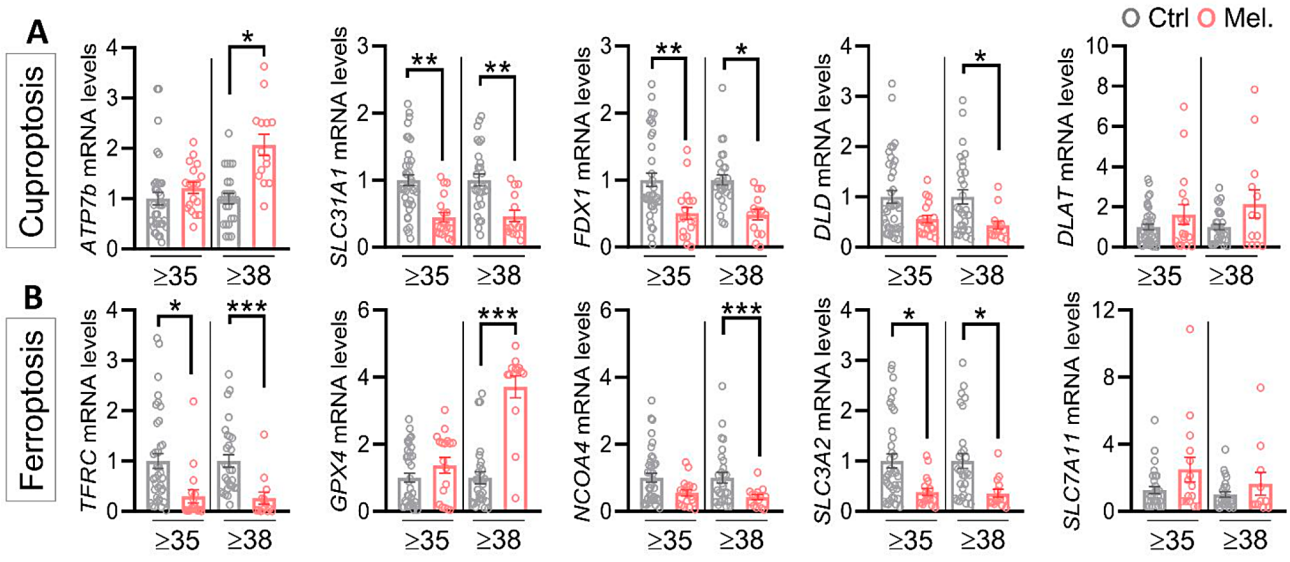


**Fig. 2** Expression levels of cuproptosis and ferroptosis-related genes in human cumulus cells following melatonin supplementation across different age groups. mRNA expression levels of genes associated with cuproptosis (**A**) and ferroptosis (**B**) were measured in human cumulus cells from participants aged ≥ 35 years and ≥ 38 years after melatonin supplementation. The bars represent fold changes in gene expression relative to the control group for each age group. Cuproptosis-related genes include ATP7B, SLC31A1, FDX1, DLD, and DLAT, while ferroptosis-related genes include TFRC, GPX4, NCOA4, SLC3A2, and SLC7A11. Data were assessed using a t-test, with significant changes observed between melatonin-treated and control groups. Genes such as ATP7B and GPX4 showed particularly pronounced age-dependent responses. Statistical significance is indicated as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

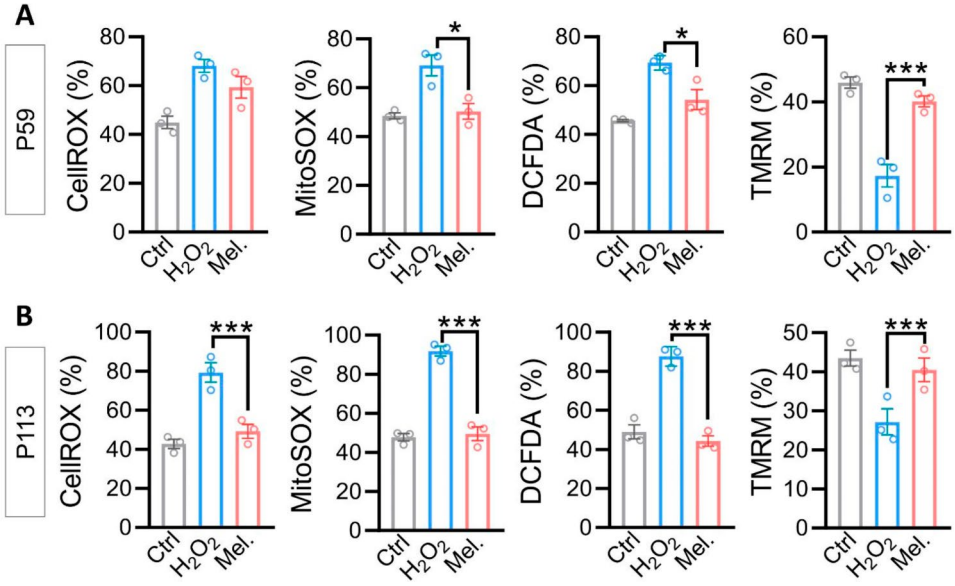


**Fig. 3** Melatonin attenuated ROS-induced mitochondrial dysfunction in aging HGL5 cells. HGL5 cells at early (P59) and late (P113) passages were treated with melatonin (25 μM) for 24 h to assess its effects on mitochondrial function. Mitochondrial function was evaluated using flow cytometry to measure four key parameters: (**A**) oxidative stress, (**B**) mitochondrial ROS levels, (**C**) hydrogen peroxide levels, and (**D**) mitochondrial membrane potential. Data are presented as percentage (%). The graph demonstrates how melatonin treatment affects these parameters differently in early versus late passage cells, highlighting its potential age-dependent effects. Statistical significance was analyzed using one-way ANOVA followed by a post hoc Tukey test to determine significant differences between groups and is denoted as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

key metabolic gene expression. Our study showed that melatonin significantly restored the expression of genes associated with glycolysis and the TCA cycle in $H_2O_2$-treated cells (Fig. 4). Melatonin normalized levels of glycolytic genes (HK2, ENO1, PKM1, LDHA) and improved expression of TCA cycle-related genes (CS, IDH1, SUCLA2, FH, MDH1). These genes contribute to enhanced ATP production. The restoration

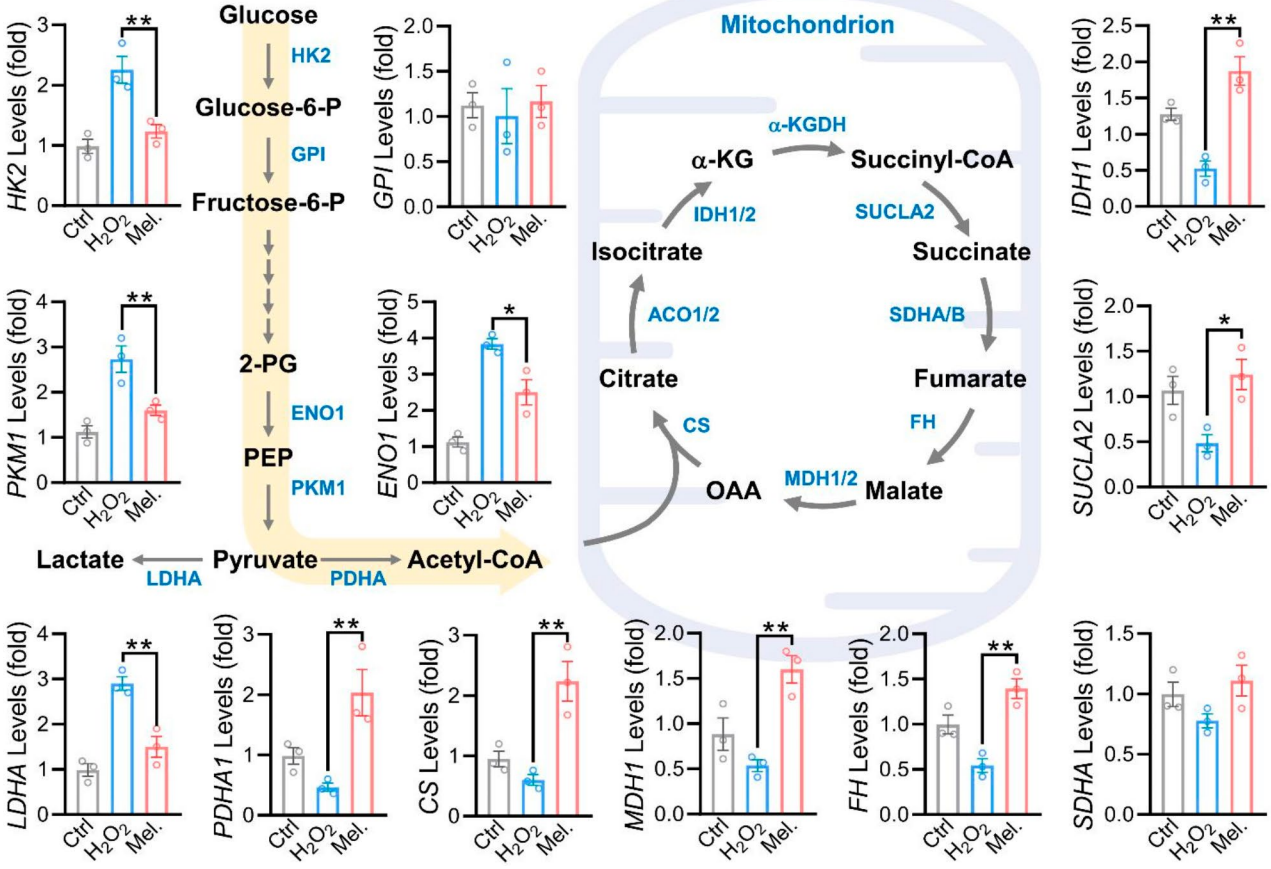


**Fig. 4** Melatonin influenced metabolic reprogramming in aging HGL5 cells. This figure illustrates the effects of melatonin supplementation on key metabolic pathways in aging HGL5 cells. The diagram presents simplified representations of glycolysis and the tricarboxylic acid (TCA) cycle, with key enzymes and metabolites highlighted. Metabolic changes were quantified, and significant differences between groups were assessed using one-way ANOVA followed by a post hoc Tukey test to determine the impact of melatonin on these metabolic pathways. Statistical significance is denoted as $^{*}p < 0.05$, $^{**}p < 0.01$

of these gene expressions suggests melatonin's potential to reprogram glucose metabolism and the TCA cycle, thereby improving the overall metabolic health of HGL5 cells under oxidative stress.

### Melatonin normalized the decrease in oxygen consumption rate under oxidative stress in aging HGL5 cells

To assess melatonin's impact on mitochondrial function in aging HGL5 cells under oxidative stress, we measured OCR using a Seahorse bioenergy analyzer. The procedure involved measuring basal OCR and responses to oligomycin, FCCP, and antimycin A/rotenone (Fig. 5A). Melatonin treatment significantly enhanced OCR compared to the $H_2O_2$-treated group, showing improvements in basal respiration, maximal respiration, and ATP production. Melatonin with $H_2O_2$ also increased non-mitochondrial respiration and proton leak compared to $H_2O_2$ alone (Fig. 5B). Protein analysis revealed that melatonin increased levels of oxidative phosphorylation complexes V, III, and II, and elevated key antioxidant proteins NRF2 and KEAP1 in aging HGL5 cells (Fig. 5C). These findings suggest melatonin enhances the antioxidant response and supports mitochondrial function under oxidative stress.

### Melatonin modulates cuproptosis and ferroptosis gene expression in aging HGL5 cells

We examined melatonin's effects on cuproptosis and ferroptosis-related gene expression in aging HGL5 cells (P113). The study specifically aimed to evaluate the efficacy of melatonin by examining its impact on the expression levels of cuproptosis-related genes (ATP7B, SLC31A1, FDX1, DLD, DLAT) and ferroptosis-related genes (TFRC, GPX4, NCOA4, SLC3A2, SLC7A11). Melatonin significantly increased expression of ATP7B, a critical cuproptosis-related gene, and mitigated oxidative stress-induced alterations in other cuproptosis-related genes (Fig. 6A). For ferroptosis-related genes, melatonin notably increased GPX4

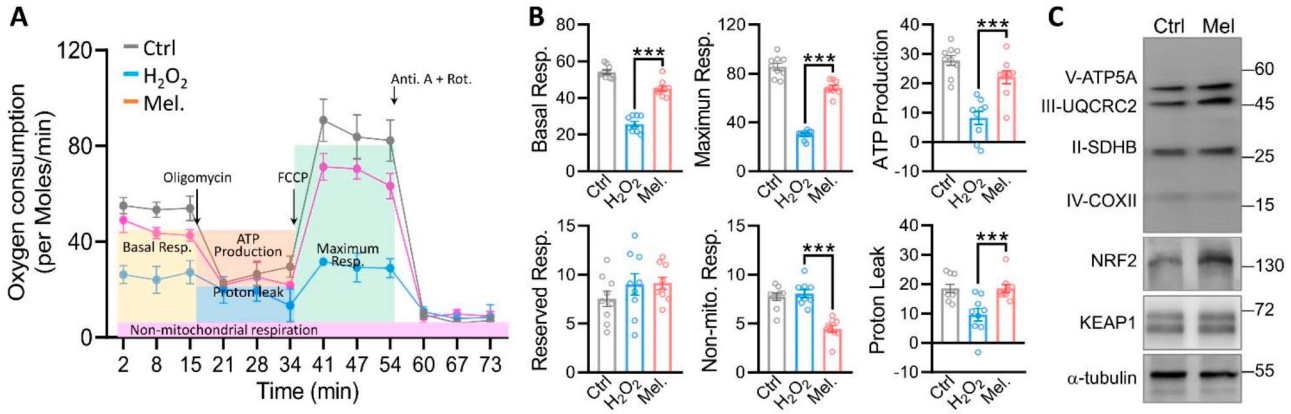


**Fig. 5** Melatonin increased mitochondrial oxygen consumption in aging HGL5 cells. This figure demonstrates the effects of melatonin on mitochondrial function in aging HGL5 cells under oxidative stress conditions. (**A**) The line graph shows real-time oxygen consumption rate (OCR) measurements using a Seahorse Bioscience analyzer. The arrows indicate the injection points of various compounds: oligomycin (1 μM), FCCP (1 μM), and antimycin A (0.5 μM) with rotenone (0.5 μM). (**B**) Bar graphs represent quantified OCR values at key stages of mitochondrial respiration: basal respiration, maximal respiration, ATP production, spare respiratory capacity, proton leak, and non-mitochondrial respiration. Statistical significance was assessed using one-way ANOVA followed by a post hoc Tukey test. (**C**) Western blot analysis was performed to evaluate the levels of oxidative phosphorylation complexes and the antioxidant proteins NRF2 and KEAP1. Statistical significance is indicated by ***$p < 0.001$

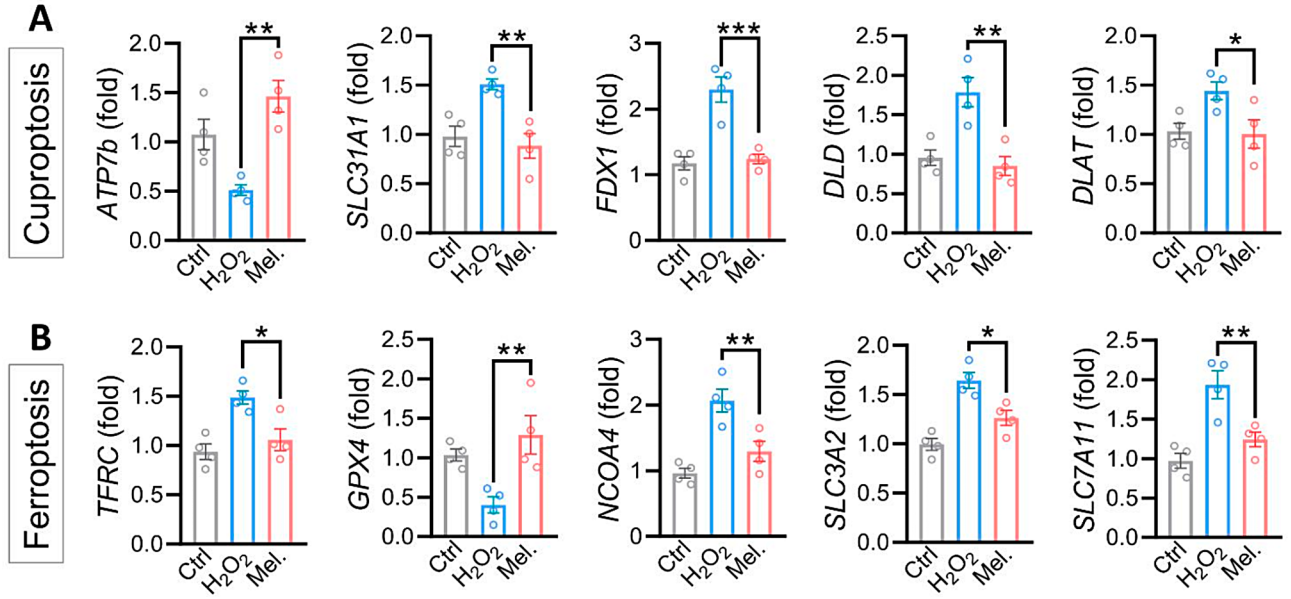


**Fig. 6** Expression levels of cuproptosis and ferroptosis-related genes after melatonin treatment in aging HGL5 cells. This figure illustrates the impact of melatonin treatment on the expression of genes involved in cuproptosis and ferroptosis pathways in aging HGL5 cells (passage 113). (**A**) Bar graphs show the relative mRNA expression levels of cuproptosis-related genes (ATP7b, SLC31A1, FDX1, DLD, DLAT) in control, $H_2O_2$-treated, and melatonin + $H_2O_2$-treated groups. (**B**) Similar representation for ferroptosis-related genes (TFRC, GPX4, NCOA4, SLC3A2, SLC7A11). mRNA levels were quantified using RT-qPCR analysis, with RNU6-1 as the reference gene. Statistical significance was assessed using one-way ANOVA followed by a post hoc Tukey test to determine differences between groups. Significance levels are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

expression while reducing other ferroptosis-associated genes (Fig. 6B). These findings suggest melatonin modulates both cuproptosis and ferroptosis pathways, providing a protective effect against oxidative stress in aging HGL5 cells.

## Discussion

This study investigated the effects of melatonin supplementation on IVF-FET outcomes in women of advanced maternal age, focusing on its impact on cuproptosis and ferroptosis in cumulus and granulosa cells. Melatonin significantly improved IVF-FET outcomes in women aged ≥ 38 years, increasing clinical pregnancy, ongoing pregnancy, and live birth rates.

Gene expression analysis of cumulus cells revealed that melatonin modulated cuproptosis and ferroptosis-related genes, with more pronounced effects in the older age group. In vitro studies using HGL5 cells showed that melatonin reduced oxidative stress markers, improved mitochondrial function, and restored expression of metabolic genes. Melatonin treatment also increased OCR and upregulated oxidative phosphorylation complexes in aging HGL5 cells. These findings suggest that melatonin supplementation may improve IVF-FET outcomes in women of advanced maternal age by modulating cuproptosis and ferroptosis pathways and enhancing mitochondrial function, potentially offering a promising adjuvant therapy for improving IVF success rates in older women.

Our study demonstrated significant improvements in IVF-FET outcomes for women aged ≥ 38 years with melatonin supplementation, including increased pregnancy and live birth rates. Previous studies have shown conflicting results regarding melatonin's effects on IVF outcomes. A double-blind randomized controlled trial (RCT) showed that melatonin supplementation in women with diminished ovarian reserve undergoing IVF increased the number of mature MII oocytes and top-quality embryos [28]. Another RCT found that melatonin supplementation for infertile women undergoing IVF treatment did not significantly improve oocyte and embryo parameters or clinical pregnancy rates [29]. However, meta-analyses have indicated that melatonin treatment significantly increased oocyte and embryo numbers and improved clinical pregnancy rates in IVF cycles [18, 19]. Unlike previous studies that rarely focused on aged infertile women and live birth rates, our research specifically addressed these aspects. We found improvements not only in oocyte and embryo numbers but also in clinical pregnancy and live birth rates following melatonin supplementation in IVF cycles for women of advanced maternal age. However, our findings warrant further investigation through large-scale RCTs. Besides, an updated systematic review and meta-analysis on antioxidants for women with ovarian aging showed significant improvements in retrieved oocytes, high-quality embryos, and clinical pregnancy rates. This analysis suggested CoQ10 might be more effective than melatonin [30]. However, direct comparisons were not made. Further research is needed to directly compare melatonin with CoQ10 and other antioxidants.

Our analysis revealed significant alterations in cuproptosis-related gene expression following melatonin supplementation, particularly in the ≥ 38 years age group. We observed upregulation of ATP7B and downregulation of SLC31A1, FDX1, and DLD. ATP7B, encoding a copper-transporting ATPase, facilitates copper efflux from cells [31]. The downregulation of SLC31A1, encoding the primary copper importer CTR1, may limit copper influx [32]. This coordinated regulation suggests melatonin's comprehensive approach to copper homeostasis, potentially reducing copper-induced cellular damage. The downregulation of FDX1 and DLD is noteworthy, as both proteins are implicated in the cuproptosis pathway, serving as targets for copper-induced protein aggregation [11, 12]. The reduced expression of FDX1 and DLD in response to melatonin may represent a protective mechanism, limiting copper-binding targets and mitigating cuproptosis initiation. Moreover, our study revealed significant alterations in ferroptosis-related gene expression following melatonin supplementation, particularly in the ≥ 38 years age group. We observed a significant increase in GPX4 expression and decreases in TFRC and NCOA4. GPX4, a key antioxidant enzyme, is considered the primary defense against ferroptosis [16, 33]. The melatonin-induced increase in GPX4 expression suggests enhanced capacity to mitigate oxidative stress and prevent lipid peroxidation, crucial for maintaining oocyte quality. This finding aligns with previous studies demonstrating decreased GPX4 expression in aging granulosa cells or ovaries [10, 34], and suggests that melatonin may counteract this age-related decline. The decrease in TFRC expression, encoding the transferrin receptor [35], may reduce cellular iron uptake, potentially protecting against iron-mediated oxidative damage. Similarly, the reduction in NCOA4 expression, a gene involved in the autophagic degradation of ferritin, known as ferritinophagy [36], further supports melatonin's role in modulating iron metabolism to mitigate ferroptosis risk.

Our findings suggest melatonin can regulate copper and iron homeostasis and protect against cuproptosis and ferroptosis in aging ovarian cells. Wang et al. discovered that melatonin supplementation can alleviate decabromodiphenyl ether-induced ovarian dysfunction in rats by reversing the expression of cuproptosis-related genes [37]. Moreover, melatonin has been shown to reduce ferroptosis in various diseases affecting multiple organs, suggesting its potential as a therapeutic agent for ferroptosis-associated conditions [38, 39]. Melatonin's influence on cuproptosis and ferroptosis likely involves multiple interconnected mechanisms. Melatonin is a potent antioxidant that directly scavenges free radicals and upregulates antioxidant enzymes. This action may protect against both cuproptosis and ferroptosis by reducing oxidative stress [40, 41]. It regulates metal homeostasis by modulating transporters and binding proteins, potentially affecting copper and iron metabolism crucial in these pathways [42, 43]. Melatonin's ability to maintain mitochondrial

function may protect against both cuproptosis and ferroptosis, which involve mitochondrial dysfunction [12, 44, 45]. Its lipid peroxidation inhibitory effects directly counteract a key feature of ferroptosis [14, 46]. Furthermore, melatonin modulates various cell death pathways, potentially influencing key proteins in cuproptosis and ferroptosis [12, 39]. This multifaceted and interconnected protective effect warrants further research to elucidate the relative contributions and potential synergies of these mechanisms in the context of ovarian aging and fertility treatment.

Moreover, our results demonstrate a significant reduction in intracellular and mitochondrial ROS levels and a restoration of mitochondrial membrane potential following melatonin treatment, suggesting that melatonin effectively mitigates oxidative stress-induced mitochondrial dysfunction. This protection is crucial in ovarian aging, as mitochondrial dysfunction is a hallmark of age-related decline in oocyte quantity and quality [47, 48]. The metabolic reprogramming observed in melatonin-treated HGL5 cells, characterized by enhanced expression of genes involved in glycolysis and the TCA cycle, indicates a shift towards more efficient energy production. The increased OCR and upregulation of oxidative phosphorylation complexes further support melatonin's role in enhancing mitochondrial function and energy metabolism in HGL5 cells. Several studies have highlighted the beneficial effects of melatonin on mitochondrial function. He et al. discovered that mitochondria in mouse oocytes synthesize melatonin, which enhances mitochondrial function by increasing mtDNA copy number, mitochondrial membrane potential, mitochondrial distribution and ATP production. Additionally, it reduces oxidative stress and improves oocyte quality and subsequent embryo development during in vitro maturation (IVM) [49]. An et al. found that melatonin supplementation during IVM of bovine oocytes reduced oxidative stress, improved mitochondrial function and spindle assembly and enhanced subsequent development and quality of cloned embryos [50]. Kandil et al. reported that melatonin supplementation during IVM and vitrification improved the developmental competence, mitochondrial distribution, and intensity of both fresh and vitrified/thawed buffalo oocytes [51]. Yang et al. demonstrated that adding 10 μM melatonin to the IVM medium improved the developmental potential of oocytes from older reproductive-aged women by enhancing mitochondrial function and reducing oxidative stress [52].

The differential effects of melatonin supplementation between women aged ≥ 35 years and those aged ≥ 38 years are noteworthy. Melatonin showed more pronounced benefits in improving pregnancy outcomes and inducing changes in cuproptosis and ferroptosis-related gene expression in the ≥ 38 years group. This age-specific response suggests that melatonin's protective effects may be particularly crucial for women of more advanced maternal age, who face higher risks of age-related decline in oocyte quantity and quality. The enhanced responsiveness in the older cohort may be attributed to cumulative oxidative stress and mitochondrial dysfunction associated with reproductive aging [6, 53]. These findings underscore the potential for tailored melatonin supplementation strategies based on maternal age in ART.

It is important to acknowledge the limitations of this study. This study was conducted as a prospective cohort study rather than an RCT, which may introduce selection bias and limit causal inference. While we observed significant changes in gene expression and cellular function, the direct causal relationship with improved IVF outcomes requires further investigation. The study's focus on a specific population may limit generalizability, and research is needed to determine efficacy in diverse patient groups and establish optimal treatment protocols. Based on our in vitro studies using HGL5 cells, it is likely that melatonin reduces oxidative stress in granulosa cells in vivo, potentially improving oocyte quality. However, the HGL5 cell line may not fully represent the characteristics of granulosa cells in vivo. Lastly, the long-term safety and potential interactions of melatonin supplementation with other fertility treatments warrant careful consideration and further investigation. Therefore, future research should focus on validating and optimizing melatonin's use in fertility treatments through large-scale, multi-center RCTs across diverse patient populations. These studies should investigate optimal dosing regimens, timing of supplementation, and potential synergistic effects with other treatments. Advanced analyses of oocytes and embryos could elucidate the precise molecular mechanisms of melatonin's effects on cuproptosis and ferroptosis pathways. Long-term studies should assess the impact on offspring health and development. Developing targeted interventions that modulate cuproptosis and ferroptosis pathways may lead to more effective fertility treatment. These directions aim to establish melatonin as a key therapeutic agent in reproductive medicine.

In conclusion, this study provides compelling evidence for melatonin's protective effects against cuproptosis and ferroptosis in aging cumulus and granulosa cells, offering new insights into its benefits for IVF outcomes in women of advanced maternal age. Melatonin's mechanisms likely involve its antioxidant properties and ability to influence mitochondrial function. Our results demonstrate that melatonin significantly

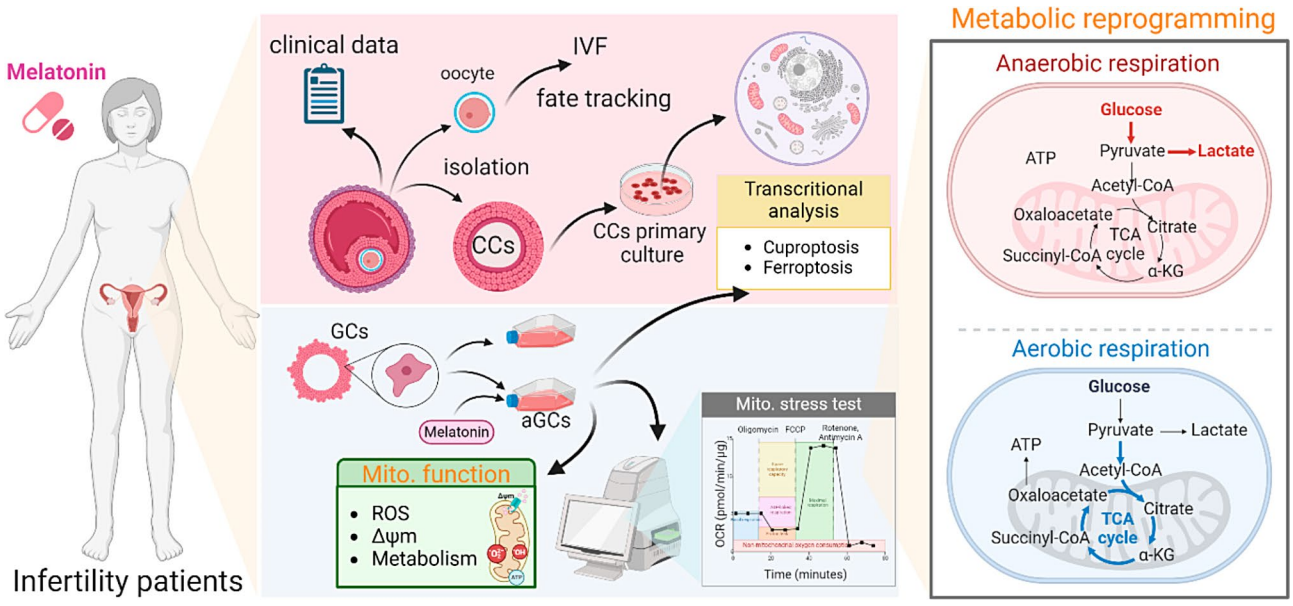


**Fig. 7** Schematic representation of melatonin-mediated reprogramming of energy metabolism in cumulus cells and granulosa cells. This diagram illustrates the key pathways and molecular mechanisms through which melatonin influences energy metabolism in cumulus cells and granulosa cells to mitigate cuproptosis and ferroptosis. It highlights how melatonin exerts protective effects on germ cell health and functionality by modulating metabolic pathways and reducing oxidative stress

enhances mitochondrial function and reprograms energy metabolism, effectively mitigating regulatory cell death triggered by metal ion metabolism imbalances in aging cells (Fig. 7). These findings contribute to our understanding of ovarian aging, opening new avenues for targeted interventions in fertility treatment. Future research should focus on elucidating precise molecular mechanisms, optimizing supplementation protocols, and evaluating long-term efficacy and safety of melatonin in fertility treatment.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-024-01311-w.

Supplementary Material 1: The clinical data for final analysis in this study, including 86 women who received melatonin supplementation prior to IVF and a control group of 75 women who underwent IVF without melatonin supplementation

Supplementary Material 2: Primer sequences designed for RT-PCR

Supplementary Material 3: The full uncropped gels and blots images

### Author contributions

K.H.T. and L.T.L. contributed to the conception and design of the study; K.H.T. and L.T.L. performed the clinical study; C.J.L. and L.T.L. performed the experimental study and the statistical analysis; K.H.T. and C.J.L. wrote the first draft of the manuscript; and L.T.L. wrote sections of the manuscript. All the authors contributed to manuscript revision and read and approved the submitted version.

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### Data availability

Data is provided within the supplementary information files.

## Declarations

### Ethics approval and consent to participate

The study followed the principles set forth in the Declaration of Helsinki for medical research involving human subjects. It also obtained approval from the institutional review board at Kaohsiung Veterans General Hospital, under the identifier KSVGH21-CT1-43. All procedures were carried out in accordance with the approved guidelines. All participants provided written informed consent.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

### Clinical trial number

This study was registered on ClinicalTrials.gov with the identifier NCT06546774 (Registration Date: August 9, 2024).

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