Effects of melatonin on specific gene expression in the ovaries of superovulated rats

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ABSTRACT

This study investigated the effects of different doses of melatonin administered with superovulation on ovarian health at biochemical and molecular levels. Four groups were created: Control (Con), Superovulation (So), Superovulation+5 mg/kg melatonin (SoM5), and Superovulation+20 mg/kg melatonin (SoM20). It was determined that there was a decrease in ovary tissue MDA levels in groups compared to the control (P<0.01). It was determined that *PGR* expression increased approximately 7-fold in SoM20 compared to the control (P<0.001). It was found that the expression level of the *COX-2* was increased in the experimental groups (So, SoM5, and SoM20) compared to the control (P<0.05). It was determined that the expressions of the *EREG* in the SoM20 increased approximately 5-fold compared to the control (P<0.001). Positive correlation was determined between serum MDA levels and *Bcl-2* and *NRF2* genes (P<0.01). A positive correlation was found between *COX-2* and *PGR* and *EREG* genes (P<0.001), between *IL-1β* and *Bcl-2* and *OSGIN1* genes (P<0.05), between *Bcl-2* gene and *NRF2* and *BMP15* genes (P<0.05), between *PGR* gene and *EREG* genes (P<0.001), and between *NRF2* gene and *BMP15* and *OSGIN1* genes (P<0.05). Protein–protein interaction network analysis identified COX-2, PGR, and EREG as central nodes within a 28 node, 213 edge network, and MCODE revealed a dense PTGER/PTGES module (score 4.00). Gene enrichment analysis demonstrated significant overrepresentation of reproductive and ovulation related pathways (FDR<0.05). In conclusion, 20 mg/kg melatonin administered during superovulation may support ovarian health and folliculogenesis by increasing the expression of the *PGR* and *EREG* genes in the ovary.

Introduction

Superovulation enhances the number of ovulated oocytes through the administration of exogenous gonadotropins. During ovulation and oocyte maturation, elevated prostaglandin synthesis induces elevated oxidative stress in the ovaries. Additionally, gonadotropin-induced oocyte maturation can provoke inflammation in ovarian tissue, further exacerbating oxidative stress and promoting lipid peroxidation. To mitigate these effects, the use of antioxidants is a widely adopted strategy (27).

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous indoleamine hormone primarily synthesized in the pineal gland. It plays critical roles in regulating circadian rhythms, supporting the immune system, and maintaining ovarian and oocyte health (45, 49). Because of its ability to dissolve in both water and lipids, melatonin easily crosses cell membranes, supporting antioxidant mechanisms in the ovary and follicles, thus playing a significant role in reproductive health. Among all known antioxidants, melatonin exerts the strongest effect on reducing oxidative stress in ovarian follicles during follicular development (45). Therefore, melatonin is considered a potent agent that protects the female reproductive system. However, limited information is available at the molecular level. Alshehri et al. (4) reported that administration of melatonin at varying doses ranging

from 5 mg to 50 mg significantly reduced oxidative stress in rats. Similarly, Gobbo et al. (20) demonstrated that long-term treatment with varying doses of melatonin decreased oxidative stress even at low concentrations. Furthermore, Atasever et al. (5) showed that chronic administration of melatonin at a dose of 20 mg/kg in rats led to inhibition of oxidative stress. Based on these findings, the present study selected melatonin doses of 5 mg/kg and 20 mg/kg for evaluation.

This study aimed to determine the effects of varying melatonin concentrations on ovarian and oocyte health to prevent oxidative damage that may occur during superovulation. This study investigated the expression of genes involved in cyclooxygenase 2 (*COX-2*), interleukin 1 beta (*IL-1β*), tumor necrosis factor-alpha (*TNF-α*), B-cell lymphoma 2 (*Bcl-2*), progesterone receptor (*PGR*), nuclear factor erythroid 2-related factor 2 (*NRF2*), bone morphogenetic protein (*BMP15*), oxidative stress-induced growth inhibitor 1 (*OSGIN1*), epiregulin (*EREG*). In addition to determination of malondialdehyde (MDA) levels in both ovarian tissue and serum, protein-protein interaction and pathway analysis were also performed.

Material and Methods

Animal and Experimental Design: Twenty-eight female Wistar albino rats (8-10 weeks old) were divided into 4 groups: Control (Con), Superovulation (So), Superovulation + 5 mg/kg melatonin (SoM5), and Superovulation + 20 mg/kg melatonin (SoM20). Each group had 7 animals, and the rats were housed in polycarbonate cages under a 12-hour light/12-hour dark cycle. The rats were kept at 21±2 ºC during the experiment and at 55% humidity. For superovulation treatment, a dose of 40 µg of gonadotropin-releasing hormone (GnRH) (Receptal, MSD Animal Health, Germany) was injected, as reported by Agca et al. (2). 300 IU/kg of pregnant mare serum gonadotropin (PMSG) (Oviser, Hipra, Spain) was administered 48 h after the GnRH injection. PMSG was injected with human chorionic gonadotropin (hCG) (Chorulon, MSD Animal Health, Germany) after 48 hours. Rats were anesthetized (80 mg/kg ketamine and 12 mg/kg xylazine) 6 h after hCG injection (2). Melatonin (M25-1G, Sigma-Aldrich, USA) was injected into the rats every evening at 5:00 PM, starting one week before the superovulation protocol. Melatonin was injected at 5 and 20 mg/kg in the SoM5 and SoM20 groups. All injections performed in this study were administered intraperitoneally.

Sample Collection: Anesthetized rats were euthanized via cardiac blood collection. Blood was collected into gel and clot activator tubes (biochemistry tubes) for serum separation. In the control group, blood sampling was performed during the estrus stage, as determined by vaginal cytology. The blood samples were centrifuged at 3000xg for 10 minutes at +4°C. After centrifugation, the supernatant was transferred to a new sterile tube and stored at -80°C until analysis. The ovaries were quickly transferred to a sterile tube and placed in liquid nitrogen to maintain the cold chain. The samples were then stored at -80°C until analysis.

Total RNA Isolation and cDNA Synthesis: Total RNA was isolated using the TRIzol method (40). For total RNA isolation, 1 mL of TRIzol™ Reagent (15596018, Thermo Fisher, USA) was added to sterile tubes, and approximately 50 mg of the ovary tissue was added, and tissue homogenization (HG-15D Daihan Scientific, Korea) was performed. After this process, the samples were kept at room temperature for approximately 10 min according to the kit protocol and then diluted with 30-50 µL of nuclease-free water according to the pellet size by passing through the chloroform-isopropyl alcohol-ethyl alcohol stages. The purity and concentration of the obtained RNAs were measured using a nucleic acid spectrophotometer. RNA integrities were also checked by agarosegel electrophoresis (100V for 25 min).

To prevent possible DNA contamination of the total RNA obtained, DNA digestion was performed according to the kit's protocol (EN0521, DNase I, RNase-free, Thermo Fisher Scientific, USA). After digestion, samples were converted to cDNA using a cDNA Synthesis Kit (4368814, High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, USA). According to the kit protocol, samples were exposed to reaction in a thermal cycler (T100, BioRad, Singapore) at 42°C for 60 min, 25°C for 5 min, and 70°C for 5 min.

Quantitative PCR Analysis: A Rotor-Gene Q device (5 PLEX HRM, Qiagen, Germany) was used to analyze *OSGIN1*, *BMP15*, *EREG*, *PGR*, *COX-2*, *NRF-2*, *IL-1β*, *TNF-α*, and *Bcl-2* genes expression levels in samples. A SYBR Green dye-containing kit (Power SYBR Green PCR Master Mix, 4368577, Thermo Fisher Scientific, USA) was used for amplification. *ACTB* (Actin Beta) was used as an internal control (30). The primer sequences used for gene amplification were generated using the literature and Primer-BLAST (NCBI) (Table 1).

Samples were subjected to the following protocol: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 30 s. The reaction was performed in duplicate. Following the reaction, melting-curve analysis was also performed.

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Table 1. Forward and reverse primer sequences of studied genes

Genes	Forward and Reverse Sequences	bp	Reference
ACTB	F:5'- GCAGGAGTACGATGAGTCCG-3'	74	(34)
	R:5'-ACGCAGCTCAGTAACAGTCC-3'		
COX-2	F:5'-TGTATGCTACCATCTGGCTTCGG-3'	94	(19)
	R:5'- GTTTGGAACAGTCGCTCGTCATC -3'		
TNF-α	F:5'- ACTGAACTTCGGGGTGATCG -3'	153	(56)
	R:5'-GCTTGGTGGTTTGCTACGAC-3'		
IL-1β	F:5'- ACAAGGAGAGACAAGCAACGAC -3'	140	(6)
	R:5'- TCTTCTTTGGGTATTGTTTGGG -3'		
Bcl-2	F:5'-TGGCCTTCTTTGAGTTCGGT-3'	111	(29)
	R:5'-GATGCCGGTTCAGGTACTCA-3'		
PGR	F:5'-CCCACAGGAGTTTGTCAAGCTC-3'	326	(50)
	R:5'-TAACTTCAGACATCATTTCCGG-3'		
NRF2	F:5'- TTGTAGATGACCATGAGTCGC -3'	141	(19)
	R:5'- TGTCCTGCTGTATGCTGCTT -3'		
BMP15	F:5'-TGATAAAGCCGTCAGCCAGT-3'	57	(48)
	R:5'-TCTGTATATGCCAAGGACCTCT-3'		
OSGIN1	F:5'-TGTGAAACCAGGAGCTGTCC-3'	110	*
	R:5'-CGAGGCCTTCGGAAAGGTAT-3'		
EREG	F:5'-TATCAGCACAACCGTGATTCC-3'	145	(40)
	R:5'-ATGCAAGCAGTAGCCGTCC-3'		

bp: Base pair, **F:** Forward, **R:** Reverse, *: Designed in this study.

Determination of Total Protein and Malondialdehyde Levels: Total protein levels in serum and ovarian tissue homogenates were measured using a BCA Assay Kit (Pierce™ BCA Protein Assay Kit, 23225, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Absorbances were measured at 562 nm using an AMR-100 microplate reader (Allsheng, CHINA).

MDA levels of serum and ovarian tissues were determined spectrophotometrically according to the reported method (33). In addition, serum melatonin levels of samples were determined by an AMR-100 microplate reader (AMR100, Allsheng, CHINA) at 450 nm via ELISA kit (EA0048Ra, BT Lab, CHINA).

Protein-protein Interaction and Network Analysis: Direct and indirect protein-protein interactions (PPI) analysis was performed using STRING. (version: 12.0) (18). The number of directly acting proteins (1st Shell) was limited to 5, and the number of indirectly acting proteins (2nd Shell) was limited to 20 (48). Apart from these limitations, the minimum required interaction score was limited to a low confidence of 0.150. STRING data was then visualized using the Cytoscape (version 3.4.0) program (34). In this process, a differentially expressed genes (DEG) network was created using the estimated PPI data. The proposed PPI network was structured using a confidence interval of >0.40. The network structure of STRING was analyzed using the MCODE 2.0.0 plugin with the default parameters (degree cutoff = 2, node score cutoff = 0.2, k-core = 2, and max depth = 100). The analysis identified central genes and important modules were detected.

Gene enrichment analysis was conducted using the STRING platform (34). Biological process annotations were retrieved from Gene Ontology (GO), and signaling pathways were mapped via the KEGG database. In this analysis, pathways with a false discovery rate (FDR) of less than 0.05 were considered statistically significant (7). The top 15 pathways most strongly associated with the study were visualized using the ggplot2 package (version 3.5.1) in R (version 2024.12.0).

Statistical Analysis: The SPSS package program (Version 24.0) was used to evaluate the data. The sample size of the study was calculated as at least 28 animals when the effect size was 0.60, the α-error was 0.05, and the power of the test was 0.80. Before performing the statistical analysis, data were examined for normality with the Shapiro-Wilk test. One-way ANOVA and Tukey's tests were performed to evaluate differences between groups. Expression levels of the studied genes were calculated with the $2^{-\Delta\Delta Ct}$ method specified by Livak and Schmittgen (29) and presented as fold change. Statistical analysis of the gene change between groups was performed with the t-test. Correlations between MDA, serum melatonin, and genes were performed using the Spearman Correlation Coefficient test.

Results

Biochemical parameters

Serum MDA levels were approximately 40 pg/ml in the experimental and control groups. No statistically significant differences were observed between the groups (Figure 1). It was determined that there was no significant difference in serum MDA levels between the experimental groups compared to the control group (P>0.05) (Figure 1b). However, a significant decrease in ovarian tissue MDA levels was observed in the experimental groups (So, SoM5, SoM20) compared with the control group (P<0.05) (Figure 1a).

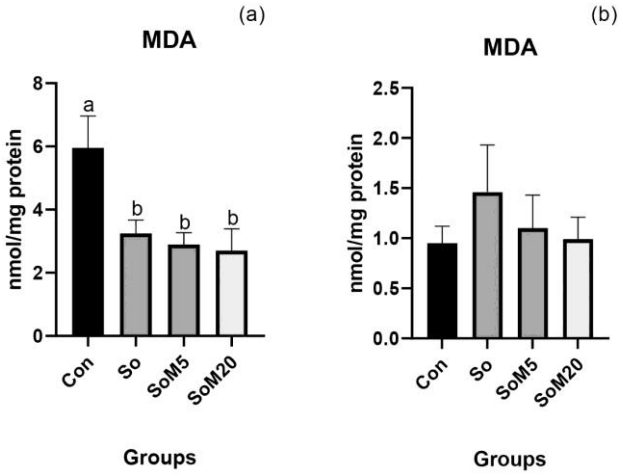

Figure 1. Malondialdehyde (MDA) levels in ovarian tissue and serum (a): MDA levels in ovarian tissue (b): MDA levels in serum Con: Control group; So: Superovulation group; SoM5: Superovulation + 5 mg of melatonin; SoM20: Superovulation + 20 mg of melatonin; SE: Standard error; different letters in the same column indicate significant differences between groups.

Expression Levels of Target Genes

The expression of the *COX-2* gene was significantly increased in the experimental groups (So, SoM5, and SoM20) compared with the control group (P<0.05; P<0.01; P<0.001, respectively). However, there was no significant up-regulation in the expression of *IL-1β, TNFα, Bcl-2, NRF2, BMP15, and OSGIN1* genes in the experimental groups compared to the control group (P>0.05). It was determined that there was an approximately 7-fold upregulation in the *PGR* gene expression level in the SoM20 group compared with the control group (P<0.001). Similarly, it was determined that there was an approximately 5-fold significant up-regulation in the expression level of the *EREG* gene in the SoM20 group compared to the control group (P<0.001) (Figure 2).

A positive correlation was determined between serum MDA levels and the expressions of the *Bcl-2* and *NRF2* genes (P<0.001; P<0.01, respectively). In addition, a positive correlation was determined between the *COX-2*, *PGR* and *EREG* genes (P<0.001). A positive correlation was determined between the *IL-1β* gene and *OSGIN1* genes (P<0.05). A positive correlation was determined between the *Bcl-2* and *BMP15* genes (P<0.01; P<0.05, respectively). Moreover, *PGR* and *EREG* genes were positively correlated (P<0.001). A significant positive correlation was also determined between the *NRF2* and *OSGIN1* genes (P<0.05) (Table 2).

Table 2. Correlation table of melatonin, MDA and gene expression results

	Melatonin	**D-MDA**	**S-MDA**	***COX-2***	***IL-1β***	***TNF-α***	***Bcl-2***	***PGR***	***NRF2***	***BMP15***	***OSGIN1***	***EREG***
Melatonin	1.00	0.068	-0.092	-0.067	0.033	0.024	0.297	-0.097	0.002	-0.031	0.134	-0.218
D-MDA		1.00	0.020	-0.380	0.086	0.169	0.265	-0.267	-0.214	0.156	0.050	-0.227
S-MDA			1.00	0.074	0.358	0.164	**0.629*****	0.205	**0.544****	0.285	0.318	-0.106
COX-2				1.00	0.088	-0.087	-0.009	**0.819*****	0.192	-0.035	-0.274	**0.635*****
IL-1β					1.00	0.113	**0.381***	0.282	0.210	0.293	**0.440***	0.094
TNF-α						1.00	0.300	0.180	0.199	0.003	0.191	0.199
Bcl-2							1.00	0.132	**0.538****	**0.425***	0.317	-0.167
PGR								1.00	0.244	-0.054	-0.239	**0.712*****
NRF2									1.00	**0.424***	**0.421***	0.041
BMP15										1.00	0.301	-0.058
OSGIN1											1.00	-0.190
EREG												1.00

D-MDA: Ovarian Tissue MDA Levels; **S-MDA:** Serum Malondialdehyde *****:P<0.05; ******:P<0.01; *******:P<0.001

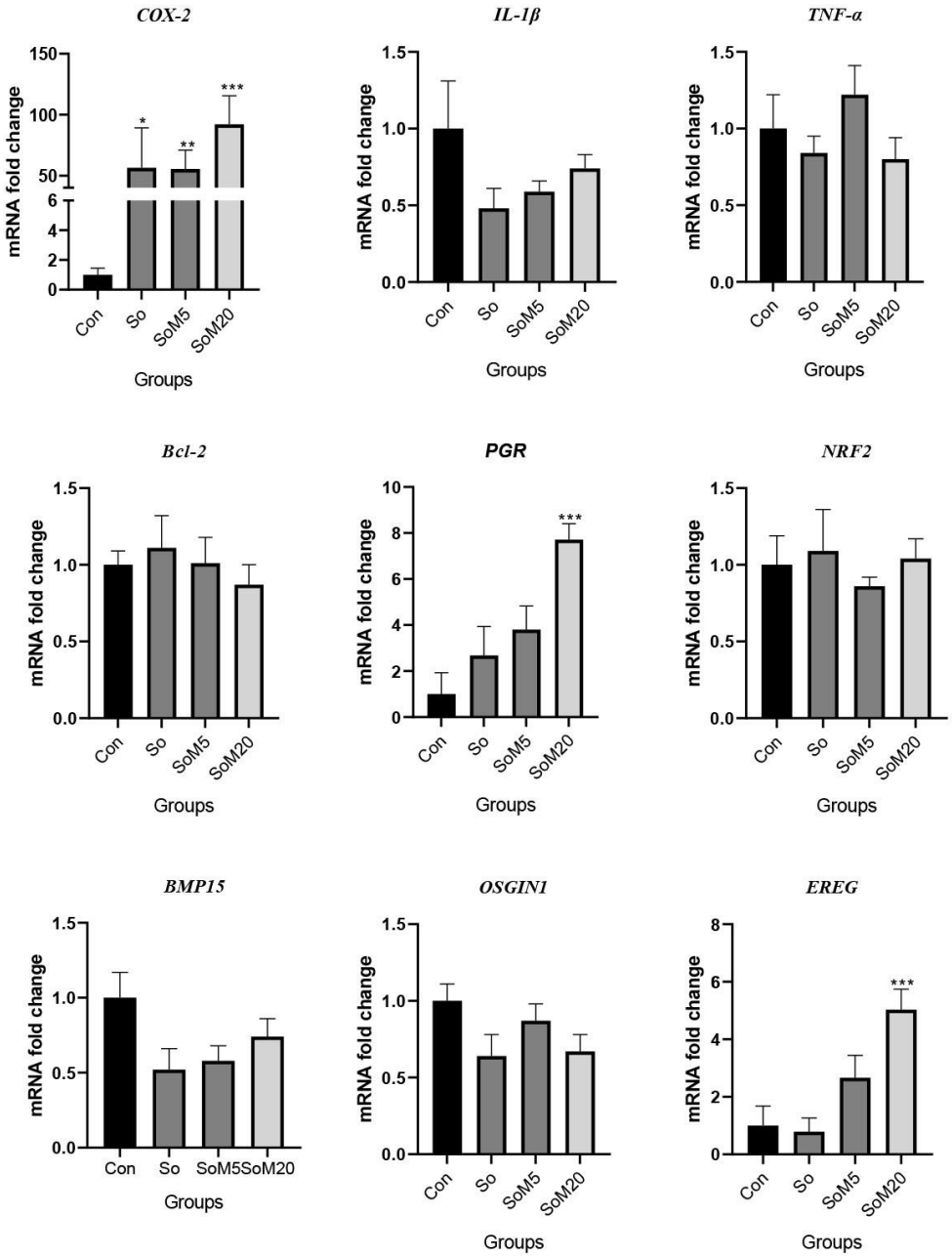

Figure 2. Expression findings of *COX-2, IL-1β, TNFα, Bcl-2, PGR, NRF2, BMP15, OSGIN1* and *EREG* genes in groups; **SE:** Standard error; *:P<0.05; **:P<0.01; ***:P<0.001

Protein-protein Interaction (PPI) and Network Analysis

Protein–protein interaction (PPI) analysis revealed that COX2, PGR, and EREG formed a connected network consisting of 28 nodes and 213 edges (Figure 3A). Among these, the highest degree of connectivity was observed within the prostaglandin E pathway genes (Figure 3B). Subsequent MCODE analysis identified a highly interconnected module composed of PTGER1, PTGER2, PTGER5, and PTGES, which contained 6 edges and exhibited an MCODE score of 4.00.

Gene enrichment analysis of the proteins interacting with DEGs revealed a pronounced overrepresentation of terms related to reproductive function. Among Gene Ontology (GO) biological processes, reproduction, ovarian follicle development, gamete generation, and ovarian steroidogenesis were significantly enriched (FDR<0.05; Figure 4). In addition, molecular function categories such as prostaglandin-E synthase activity and hormone activity reached significance, underscoring the importance of steroid and prostaglandin signaling in folliculogenesis. KEGG pathway analysis further highlighted the GnRH signaling pathway, pituitary gonadotropin complex, and ovulation cycle process as key enriched cascades (FDR <0.05).

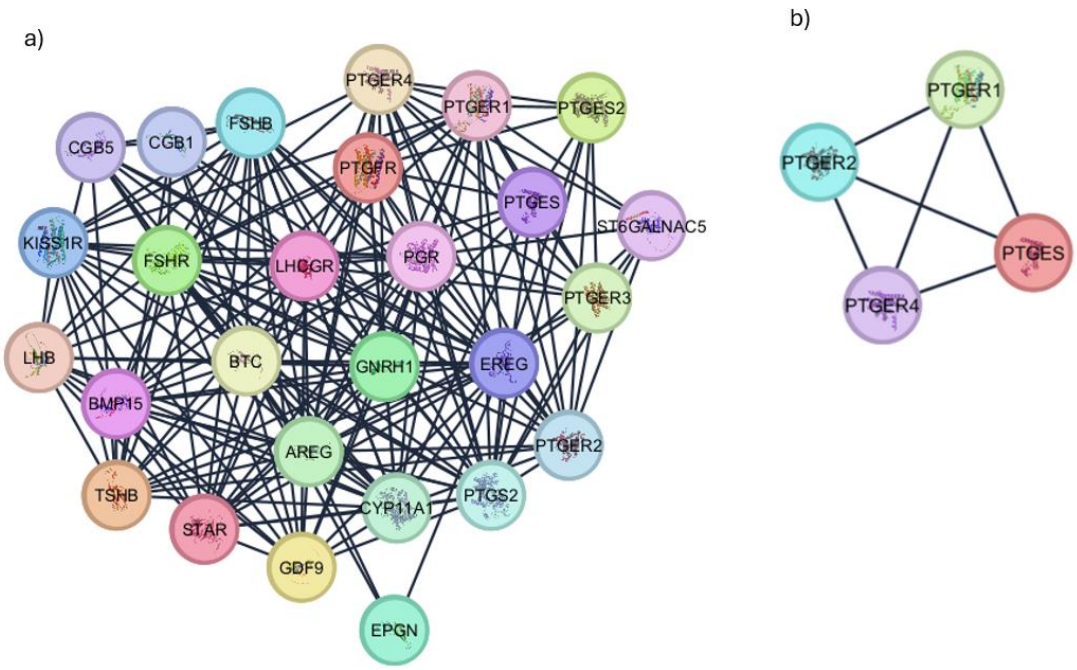

Figure 3. a) PPI network of COX-2, PGR, and EREG (28 nodes, 213 edges); **b)** MCODE module of PTGER1, PTGER2, PTGER5, and PTGES (MCODE Score 4.00).

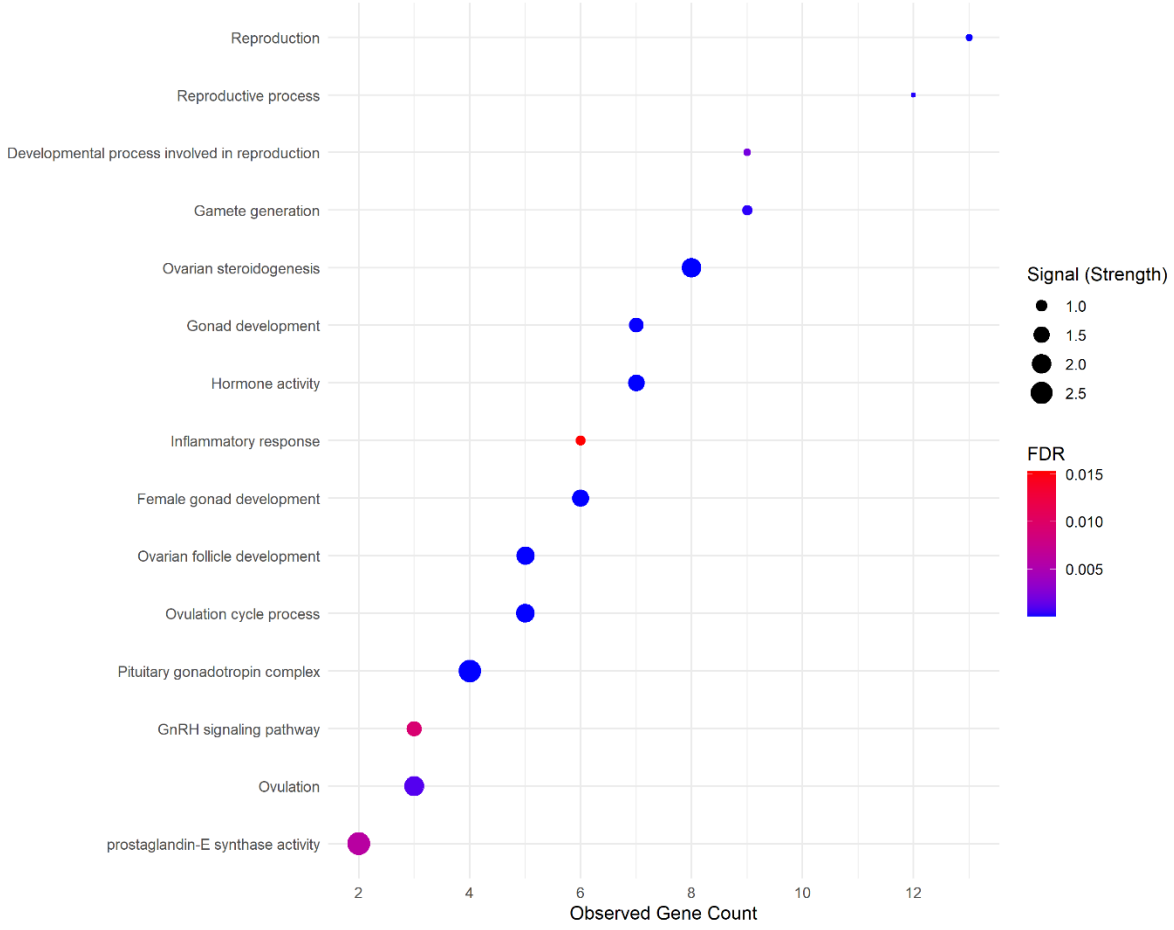

Figure 4. Functional enrichment analysis of interacting genes.

Discussion and Conclusion

In mammals, inflammation develops in the ovarian tissue during normal oocyte development. Oxidative stress also occurs due to inflammation (27). Multiple follicles stimulated by superovulation may develop and cause increased oxidative stress in the ovarian tissue. Various studies have reported that superovulation may cause oxidative stress in the ovary (17, 26). The use of various agents to prevent oxidative stress that may occur with superovulation has been the subject of many studies (9, 27). Melatonin, an endogenous hormone, is an important hormone that plays a role in maintaining homeostasis and reducing oxidative damage in many tissues and organs (45). There is limited molecular knowledge and studies on the effects of melatonin on the prevention of oxidative damage that may occur due to superovulation in ovarian tissue.

Melatonin is an important biological function, such as antioxidant, anti-inflammatory, and anti-apoptotic effects (45). The antioxidant properties of this hormone are higher than those of other known antioxidants. Therefore, melatonin contributes to the reproductive system by protecting the health of the ovary, oocyte, and gamete (17). The effects of exogenous melatonin administration on serum melatonin concentrations are controversial. Different studies have reported different results depending on factors such as dose, duration of administration, and sampling time (44, 45, 54). In addition, a study by Yeleswaram et al. (52) reported that the effect of oral melatonin administration was greater than that of intravenous melatonin administration. Terrón et al. (46) found that the administration of exogenous melatonin to rats at a dose of 20 mg/kg for 15 weeks had no significant effect on serum melatonin levels. The administration of exogenous melatonin at doses of 5 and 20 mg/kg had no significant effect on serum melatonin levels. This study and similar findings in the literature suggest that the effect of exogenous melatonin administration may depend on variables such as dose, duration of administration, and sampling time.

The balance between oxidant and antioxidant production is important during follicular development. When this balance is disrupted, follicular and ovarian health is negatively affected, leading to ovarian damage (49). Although it was determined that ovarian MDA levels were significantly reduced in the superovulation groups compared with the control group, there is no reference range for normal MDA levels in the ovarian tissue of rats. Studies have reported that the amount of MDA in ovarian tissue ranges from 4 to 10 nmol/mg protein in healthy control groups (6, 30, 39). Although ovarian MDA levels in the superovulation groups were lower than those in the control group, the ovarian MDA levels in the control group were not higher than those reported in similar studies in the literature. In studies in which exogenous gonadotropins were administered, the amount of MDA in ovarian tissue was reported to be approximately 10-20 nmol/mg protein (16, 30). In this study, the lower ovarian MDA levels in the So, SoM5, and SoM20 groups compared with the control group suggest that exogenous gonadotropin administration may have a protective role against damage that may occur in the ovary, but new studies are needed to better understand the effects that may occur as a result of melatonin administration.

Prostaglandins, key mediators of inflammation, play a central role in ovulation, with *COX-2* being essential for oocyte maturation, follicle rupture, and ovulation induction (3, 19). In this study, *COX-2* expression was significantly upregulated in the superovulation groups, with upregulation primarily driven by hCG injections, consistent with previous findings (2, 11). Melatonin, known for supporting folliculogenesis and regulating gonadotropin secretion, appears to modulate *COX-2* expression in a dose-dependent manner. Since COX-2 plays a role in oxidative stress and inflammatory pathways, melatonin, as a potent antioxidant, may support ovarian health by reducing COX-2-mediated oxidative damage. Although exogenous gonadotropins stimulate prostaglandin synthesis and *COX-2* expression, melatonin may mitigate inflammation and oxidative stress (44, 36). These results highlight the complex interactions between melatonin, *COX-2*, and superovulation and warrant further investigation.

PGR is a transcription factor required for ovulation in the female reproductive system because it controls oocyte release from the ovary and ovulation, and its expression increases intracellularly in parallel with the increase in progesterone (36). In rodents, the LH surge before ovulation temporarily triggers the expression of *PGR* gene (19). A previous study reported that ovulation success in folliculogenesis stimulated by gonadotropins was associated with this gene (3). However, another study reported that in the event of ovarian damage, there was a decrease in the total number of follicles but no change in the expression of the *PGR* gene (23). In other studies, this gene was found to be upregulated following superovulation (2, 11). In addition, melatonin triggers *PGR* gene expression in ovaries (15). In a study by Ezzati et al. (15), the amount of *PGR* gene mRNA expression in the ovary increased approximately 5-fold depending on the duration of melatonin application. The upregulation in the mRNA level of the PGR gene in the ovarian tissue of the SoM20 group may be related to the melatonin dose.

EREG acts in mammals in vitro by inducing oocyte maturation and cumulus cell expansion, depending on the LH signal. Following the LH surge, EREG is secreted by mural granulosa cells and acts as a paracrine mediator in cumulus cells (41). In the final stage of folliculogenesis,

EREG is upregulated (41). Increased *EREG* expression is important for healthy ovulation. Various studies have reported that *EREG* expression in ovarian tissue is rapidly but temporarily triggered by gonadotropins (14, 41). In this study, superovulation did not affect the expression of this gene. These findings suggest that the application of exogenous gonadotropin could have an impact on folliculogenesis.

Although studies have reported that melatonin has no dose-dependent effect on *EREG* expression, some have reported that melatonin induces the expression of the *EREG* gene (12, 51). Similarly, in this study, melatonin administered at a dose of 20 mg/kg significantly increased *EREG* gene expression levels in ovarian tissue by approximately 5-fold. These findings suggest that melatonin exerts a positive effect on ovulation and oocyte health through the expression of this gene.

The positive correlation between serum MDA levels and the *Bcl-2* and *NRF2* genes indicated that serum damage triggers apoptosis and antioxidant mechanisms (43). Although there is no clear study on the correlation between serum MDA levels and *Bcl-2* and *NRF2* genes, studies have reported that the expressions of these genes upregulate due to the increase in ROS levels (1, 38). The positive correlation between serum MDA levels and *NRF2* expression was also consistent with the existing literature. Various studies have reported that the expression of this gene increases in cases of ovarian damage (16, 43).

The correlation between *Bcl-2* and *NRF2* genes indicates that these genes positively affect each other during cell survival. The correlation between the anti-apoptotic gene *Bcl-2* and the *NRF2* gene, which plays a central role in cell antioxidant mechanisms, is consistent with literature studies (43). *BMP15* positively affects ovarian health and oocyte development by preventing the apoptosis mechanism (28). The correlation between the anti-apoptotic gene *Bcl-2* and the *BMP15* gene shows that these genes act together in ovarian health and support folliculogenesis.

Although no study has indicated a correlation between *NRF2* and *BMP15* expression, some studies have reported that the expression levels of both genes increase during the protection of oocyte health during folliculogenesis (23, 53). Increased oxidative stress in cells triggers the expression of *OSGIN1* via the NRF2 signaling pathway. However, this may vary depending on the tissue (25, 50). Komuczki et al. (25) reported that the NRF2 signaling pathway is activated in parallel with an increase in the amount of ROS in the ovary, and accordingly, the expression level of the *OSGIN1* gene increases.

The correlation between *COX-2*, *PGR*, and *EREG* indicates that these genes interact together during folliculogenesis and ovulation. An increase in ROS levels and prostaglandin synthesis in ovarian tissue induces the *COX-2* gene. The increase in the expression of this gene helps to reduce ROS levels in ovarian tissue by triggering the *PGR* gene (Park et al., 2020). Studies have reported that the expression of the *COX-2* and *PGR* genes increases together in ovarian tissue (41, 42). The findings obtained from these studies reveal that the *COX-2* gene, which is induced by the increase in PGE2, and the PGR genes that are involved in the formation of luteinization are positively correlated. Park et al. (37) reported that *COX-2* in ovarian tissue can trigger the expression of genes belonging to the EGF family. The fact that the *COX-2* gene triggers the expression of the *EREG* gene, which plays an active role in ovarian tissue and folliculogenesis, indicates that it plays a role in triggering ovulation.

During superovulation, luteinizing hormone (LH) acts as a paracrine stimulant by triggering the synthesis of prostaglandin E2 (PGE2) in ovarian cells. LH induces prostaglandin synthesis by increasing COX-2 activity in granulosa cells. PGE2 triggers the expression of growth factors, such as epidermal growth factor (EGF). Similarly, progesterone also triggers PGE2 synthesis. Therefore, it is thought that follicular development and healthy ovulation processes are regulated by PGE2 (24). Exogenous gonadotropin application can affect follicular development and ovulation in the ovary by triggering the expression of progesterone and PGE2 expression *PTGES*, PTGERs (24). In studies conducted on knockout mouse models, it has been reported that ovulation and follicular development do not occur in *COX-2*$^{(-/-)}$ and *PTGER2*$^{(-/-)}$ conditions (13, 21). It has been suggested that PTGER receptors support follicular growth by transmitting PGE2 signals in follicles and play an important role in this process. PGE2 has four different receptors, namely *PTGER1, PTGER2, PTGER3,* and *PTGER4,* and it has been shown that these receptors are expressed in different follicle cells. In particular, the *PTGER1, PTGER2,* and *PTGER4* genes have been reported to play active roles in the differentiation of ovarian epithelial cells (10). In addition, Nuttinck et al. (32) reported that PTGES triggers *PTGES1* and *PGE2* expression through *PTGS2* during the maturation of bovine oocytes. Exogenous gonadotropin administration triggers *PTGES* expression in the follicles and granulosa cells of the ovary (8). Trau et al. (47) suggested that exogenous gonadotropin administration has therapeutic potential for ovulation by targeting the PTGER1, PTGER2, and PTGER4 receptors. Similarly, protein-protein interaction (PPI) analysis determined that *PTGER1, PTGER2,* and *PTGER4* may play critical roles in follicle development and ovulation. These data demonstrate that PGE2 and related receptors play fundamental roles in the regulation of follicular development and ovulation processes.

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Melatonin at a dose of 20 mg/kg has been found to protect ovarian and oocyte health due to its effects on *PGR* and *EREG* genes. However, further studies are needed to determine the effects of long-term melatonin use on ovarian and oocyte health. Protein-protein interaction studies conducted in this study revealed that the PTGER1, PTGER2, PTGER4, and PTGES proteins, which are key players in prostaglandin synthesis throughout folliculogenesis and ovulation, play a central role (31). In future studies, a more detailed examination of these genes may provide additional insights into follicle development.

As a conclusion, our gene enrichment analysis revealed that melatonin-responsive genes are markedly enriched in pathways governing anti-inflammatory defenses and ovulatory regulation. Notably, this finding aligns with Jiang et al. (22), who reported that an initial bolus of melatonin disrupted normal ovulatory patterns, whereas Pacchiarotti et al. (35) demonstrated that continuous melatonin supplementation enhanced ovulatory outcomes. Taken together, these data suggest that melatonin administration may attenuate oxidative stress during superovulation and thereby improve ovulatory efficiency and protocol success.

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Ethical Statement

This study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee with decision number 2022/05-07.

Conflict of Interest

The article's authors declare that they have no conflict of interest.

Author Contributions

A.Y. conceived and investigated the study. A.Y. supervised the study. S.D. collected samples and performed RNA isolation, gene expression, MDA analysis, and protein and ELISA experiments. A.Y. and H.Ö. analyzed the results. A.Y., H.Ö. and S.D. wrote the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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