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ORIGINAL ARTICLE

Melatonin improves the in vitro growth of bovine oocytes collected from early antral follicles by maintaining oocyte-cumulus cell communication

Md Nuronnabi Islam[1,2,3] | Fumio Ebara[1,2] | Toshihiro Konno[2,4] | Hideki Tatemoto[2,4] | Ken-ichi Yamanaka[1,2]

[1]Faculty of Agriculture, Saga University, Saga, Japan

[2]The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima, Japan

[3]Department of Animal Science, Bangladesh Agricultural University, Mymensingh, Bangladesh

[4]Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan

Correspondence
Ken-ichi Yamanaka, Faculty of Agriculture, Saga University, 1 Honjo-machi, Saga 840-8502, Japan.
Email: kyama@cc.saga-u.ac.jp

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Abstract

Purpose: In vitro, oocyte development is susceptible to oxidative stress, which leads to endoplasmic reticulum (ER) stress. This study investigated whether the antioxidant melatonin attenuates ER stress and maintains oocyte-cumulus cell communication during the in vitro growth (IVG) of bovine oocytes.

Methods: Oocyte-granulosa cell complexes (OGCs) were harvested from slaughterhouse-derived ovaries and grown in vitro for 5 d at 38.5°C in 5% CO_2 humidified air. Melatonin (10^{-7}, 10^{-9}, or 10^{-11} M) was added to the culture medium.

Results: Oocyte diameter increased on day 5 from its initial value in all groups. The antrum formation rate was significantly higher in the 10^{-9} M melatonin-treated group than in the control. The melatonin-treated group showed reduced oxidative stress and increased gap junction communication compared with the control. ER stress-related genes in OGCs were significantly downregulated in the 10^{-9} M melatonin-treated group compared with those in the control. No significant changes were found in subsequent maturation among groups; however, 10^{-9} M melatonin treatment during IVG and IVM increased the maturation rate compared with that in the control.

Conclusions: Melatonin reduces oxidative stress, which attenuates ER stress in OGCs during IVG of bovine oocytes and may improve IVG efficiency in assisted reproductive technology.

KEYWORDS
endoplasmic reticulum stress, gap junction, in vitro growth, melatonin, oxidative stress

1 | INTRODUCTION

The developmental competence of oocytes grown in vitro to resume meiosis and produce blastocysts is impaired by various stressors.[1] The endoplasmic reticulum (ER) represents a vital organelle that plays a crucial role in energy metabolism, including Ca^{2+} homeostasis and redox equilibrium as well as the protein folding and maturation processes.[2] The build up of unfolded proteins in the ER lumen causes ER stress, and the unfolded protein response (UPR) activates the repair of protein folding defects.

Under severe or prolonged stress conditions, the UPR fails to resolve these defects, thereby causing the activation of proapoptotic signaling pathways in cells.[3,4] The redox balance of ER is disrupted when disulfide bonds form in the lumen of the ER. ER stress and reactive oxygen species (ROS) accretion can result from the dysregulated disulfide bond formation and rapture,[5] which, in turn, can cause mitochondrial malfunction and elevated ROS generation within the mitochondria. ER and oxidative stress escalate one another in regenerative feedback cycle which disrupts cellular processes and triggers proapoptotic signals.[5]

The pineal gland produces melatonin (N-acetyl-5-methoxy tryptamine), a multifunctional bioactive amine that acts on the target tissues. Melatonin plays various roles in all stages of development from embryo to adults.[6] Melatonin mediates the ovarian follicular cycle by acting on the ovary.[7] Melatonin in follicular fluid influences granulosa cells, steroidogenesis, and follicular function in various species.[8–11] Bovine follicular fluid contains about 10^{-11} M endogenous melatonin.[12] Melatonin is found in high concentrations in human folliclar fluid, rising propotionately with follicle size.[13] However, the reason for the high concentration of melatonin in follicles remains unexplained. Melatonin is an important antioxidant in the follicular fluid that shields granulosa cells and oocytes from oxidative damage.[14] As oocyte quality determines the success of assisted reproductive technology in infertility treatment, clinical applicaton of melatonin in women with infertility has led to improved outcomes.[15–17] Melatonin also decreases the ROS levels in oocytes, which promote in vitro maturation (IVM).[14] In mice, melatonin protects granulosa cells from induced oxidative stress, reduced apoptosis, and mitochondrial dysfunction.[18] Exogenous melatonin supplementation promotes IVM and the developmetal competence of oocytes in different species through antioxidative and antiapoptotic responses.[19–26]

Melatonin enhances the functions of mitochondria and shields them from oxidative damage as a sturdy free radical scavenger.[27,28] Melatonin downregulates the proapoptotic genes *p53* and *BAX* and upregulates the antiapoptotic gene *BCL2* and antioxidant genes *SOD1* and *GPx4* in SCNT and parthenogenetic embryos.[25,29] Therefore, melatonin may also reduce ER stress. Nonetheless, as far as we are aware, the action mechanisms of melatonin during in vitro growth (IVG) of oocyte-granulosa cell complexes (OGCs) have not yet been investigated. Thus, this research was to ascertain how melatonin affects the growth and associated parameters, such as antrum formation, oxidative stress, oocyte-cumulus communication through gap junctions and transzonal projections (TZPs), ER stress-related gene expression, and subsequent maturation, of bovine OGCs in vitro.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Unless otherwise specified, all chemicals and media were procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | OGC collection

OGC complexes were collected as previously delineated,[30] with certain modifications. We obtained bovine ovaries from a nearby slaughterhouse within about 3 h of slaughter. To prepare ovarian cortical slices, the ovaries were washed a minimum of three times in a saline solution containing 100 μg/mL streptomycin (streptomycin sulfate; Meiji Seika Pharma Co., Ltd) and 100 IU/mL penicillin (benzylpenicillin potassium; Meiji Seika Pharma Co., Ltd., Tokyo, Japan), after which the ovarian cortex was cut carefully using forceps and a surgical blade (no. 10; Feather Safety Razor Co., Ltd., Osaka, Japan), and cortical slices were made. Early antral follicles with a diameter of 1.5–1.8 mm were collected in medium 199 augmented with 0.85 mg/mL sodium bicarbonate, 2.5% (v/v) HEPES buffer solution (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 0.1% polyvinyl alcohol, and 10 μg/mL gentamycin (gentamycin sulfate solution). OGCs were collected by rupturing the follicular wall with forceps and surgical blades (No. 11; Feather Safety Razor Co., Ltd.). OGCs that had oocytes measuring 105–115 μm in size (excluding zona pellucida) were used in the experiments.

2.3 | IVG culture of OGCs

OGCs were cultured in 96-well plates for 5 d in a humidified atmosphere with 5% CO_2 at 38.5°C. Each well contained 200 μL of growth culture medium. Minimum essential medium alpha (Thermo Fisher Scientific, Waltham, MA, USA) was used as the basic growth culture medium. It was supplemented with 4% (w/v) polyvinylpyrrolidone (molecular weight 360000), 4 mM hypoxanthine, 5% (v/v) fetal bovine serum (FBS); (Thermo Fisher Scientific), 0.05 μM dexamethasone, 50 μM IBMX (AdipoGen Life Sciences, CA, USA), 100 ng/mL 17β-estradiol, 10 ng/mL androstenedione, and 10 μg/mL gentamicin sulfate (Nacalai Tesque, Kyoto, Japan).[31] To examine the effects of melatonin, 10^{-7}, 10^{-9}, or 10^{-11} M melatonin (Calbiochem, San Diego, CA, USA) was added to the basic culture medium. Day 0 was designated as the day that oocytes were collected and on day 3, a fresh medium was used to replace half (100 μL) of the culture medium. Oocytes that developed cytoplasmic degeneration with cumulus detachment were not included in subsequent experiments. Oocytes that had one or more dome-like structures or antrum like spaces along with well-attached cumulus cells were defined as healthy oocytes. IVM was conducted on healthy OGCs on the fifth day of IVG. During IVG, the oocyte diameter, antrum development, and survival were measured.

2.4 | Oocyte diameter measurement

The oocyte diameter (avoiding the zona pellucida) was recorded using a Motic Plus camera (Moticam; Shimadzu RIKA Corporation, Tokyo, Japan) and ImageJ software (National Institutes of Health, Bethesda, MD, USA) on days 0 and 5 of IVG. The OGCs were denuded using a

bore pipette on day 5 to obtain their exact diameters. In total, 115 oocytes were used in this experiment.

2.5 | IVM

On the fifth day following IVG, healthy OGCs underwent IVM, which was performed in bicarbonate buffered M-199 medium (Thermo Fisher Scientific), enriched with 5% (v/v) FBS, 0.02 IU/mL follicle-stimulating hormone (Kyoritsu Seiyaku, Tokyo, Japan), and 10 μg/mL gentamicin (Nacalai Tesque). The medium was covered with liquid paraffin (Nacalai Tesque), and the experiment was conducted at 38.5°C for 22 h in a humidified atmosphere with 5% CO_2. Subsequent to IVM, the oocytes were stripped of their surrounding cells using a small-bore pipette. To check how far along the oocyte's meiotic process was, it was fixed in aceto-ethanol for 48 h and then stained with aceto-orcein. To investigate the effect of melatonin on IVM and assess maturation competence, melatonin-treated IVG oocytes were allowed to mature under similar concentrations. In total, we used 224 oocytes for this study.

2.6 | Evaluation of oxidative stress and reduced glutathione (GSH) levels

Oxidative stress was assessed by quantifying the intracellular ROS. On the fifth day, OGCs underwent mechanical denuding and then incubated for 15 min in polyvinylpyrrolidone (PVP)-phosphate-buffered saline (PBS) with 10 μM CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA), at 38.5°C in a humidified atmosphere with 5% CO_2. The CM-H2DCFDA fluorescence dye was used to detect ROS. After washing twice with PVP-PBS, the oocytes were put in a glass-bottomed dish. The fluorescence emission was observed and recorded using a microscope (EVOS® FL; Thermo Fisher Scientific).

On the fifth day of IVG, the GSH values in the oocytes were recorded. OGCs underwent mechanical denuding and then incubated for 15 min in PVP-PBS with 10 μM CellTracker™ Blue CMF2HC (Invitrogen), at 38.5°C in a humidified environment with 5% CO_2. After washing twice with PVP-PBS, the oocytes were put in a glass-bottomed dish (Matsunami Glass Ind., Osaka, Japan). The fluorescence emission was observed and recorded utilizing the same fluorescence microscope as mentioned above. Using ImageJ, fluorescence intensity of the pictures was calculated for further analysis. In total, we used 122 oocytes for this experiment.

2.7 | RNA isolation and qRT-PCR

Following the manufacturer's guidelines, the entire RNA was isolated from OGCs collected on the fifth day of IVG by applying the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). With a few changes, the procedure was followed as outlined by Khatun et al. (2020).[32] Each replication utilized a minimum of 12 OGCs with at least three repetitions conducted. The isolated RNA was incontinently reverse transcribed to cDNA applying SuperScript® VILO™ MasterMix (Invitrogen) under the manufacturer's guidelines. Real-time PCR of cDNA samples, examined in duplicate, was conducted using the LightCycler® 96 System (Roche, Basel, Switzerland) utilizing SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR (20 μL) reaction consisted of 10 μL Supermix, 2 μL cDNA, 6 μL nuclease-free water, and 1 μL of both of the forward and reverse primers (10 μM) of the target gene. *RN18S1* was performed as an intrinsic reference gene. The qRT-PCR protocol included a denaturation phase (95°C for 30 s), followed by 40 cycles of two-step amplification (95°C for 10 s and 60°C for 20 s), and concluded with a melting curve analysis (65–97°C with a heating rate of 0.3°C, continuous fluorescence monitoring, and subsequent cooling to 30°C). The $2^{-\Delta\Delta CT}$ method was applied to determine the fold changes in the expression of the target genes relative to the expression of the endogenous reference gene *(RN18S1)*. Table 1 shows the primer sequences employed for qRT-PCR.

2.8 | Exposure of OGCs to H_2O_2

To examine the role of melatonin in relieving oxidative stress, bovine OGCs were divided into four culture groups, which were cultured for 2 h: control, only IVG medium; H_2O_2, IVG medium with H_2O_2 (100 μM, an inducer of oxidative stress); H_2O_2 + melatonin, IVG medium with H_2O_2 and melatonin (10^{-9} M); and melatonin, IVG medium with melatonin (10^{-9} M). Oxidative stress was subsequently evaluated by quantifying the intracellular ROS levels following the procedure described above. Fluorescence intensity was measured and analyzed to determine whether melatonin supplementation decreased H_2O_2-induced oxidative stress in OGCs.

2.9 | Detection of oocyte-cumulus gap junction communication (GJC)

Gap junctions facilitate the transfer of nutrients and metabolites received by the granulosa cells to the oocytes, which play pertinent roles in oocyte development.[33] GJC between the cumulus cells and the oocyte during IVG was assessed through the transfer of Calcein-AM (Cellstain®—*Calcein*—AM; DOJINDO, Kumamoto, Japan) dye from the cumulus cells to the oocyte, as outlined by Khatun et al. (2024), with certain changes.[34] Briefly, OGCs treated with or without melatonin during IVG were incubated in calcein-AM solution (1 μM) for 15 min. Subsequently, they were moved to a stain-free IVG medium for 25 min at 38.5°C in a humidified environment with 5% CO_2 to facilitate the transfer of dye from the cumulus cells to the oocyte. Oocytes were rinsed thrice in 0.1% polyvinyl alcohol (PVA)-PBS to eliminate any unincorporated dye and mounted on glass slides. Cumulus-oocyte GJC was assessed by quantifying the calcein stain in the oocyte that was transported from the cumulus cells across gap junctions via passive diffusion using quantitative fluorescence microscopy and was analyzed using ImageJ to quantify the fluorescence intensity of oocytes.

TABLE 1 Primer information.

Gene	Primer sequence (5′–3′)	Product size (bp)
GRP78	F: tgcagcaggacatcaagttc R: tttgtttgcccacctccaac	91
XBP1	F: tagcagctcagactgccaga R: attccccttggtctctgctt	215
ATF4	F: ggccaagcacttcaaacatc R: aagcatcctccttgctgttg	110
ATF6	F: tgaacttcgaggatgggttc R: gaatttgagccctgttccag	117
PERK	F: ggctgaaagatgacagcaca R: agaactggctctcggatgaa	195
IRE1	F: ccgaagttcagatggcattc R: tctgcaaaggctgatgacag	108
CHOP	F: gcaccaagcatgaacagttg R: atcgatggtggttgggtatg	116
RN18S1	F: aaacggctaccacatccaagg R: gcggaaggatttaaagtggactc	138

2.10 | Assessment of TZPs in oocytes

TZPs are thin and threadlike cytoplasmic extensions of actin filaments that emanate from cumulus cells and penetrate through the zona pellucida to reach the oocyte surface, serving as a framework for adherens junctions and gap junctions.[35,36] To evaluate the quantity of TZPs in oocytes, actin filaments were stained according to prior protocols, with certain changes.[34,37] Briefly, OGCs underwent mechanical denuding and were washed with 0.1% (w/v) PVA-PBS, followed by fixing in 4% paraformaldehyde in PVA-PBS for 60 min. Following fixation, the oocytes underwent PVA-PBS washes twice and were incubated at 4°C overnight in PVA-PBS supplemented with 0.1% bovine serum albumin (BSA-PVA-PBS). The next day, the oocytes were incubated with Acti-stain™ 488 Phalloidin (100 nM; Cytoskeleton, Inc., Denver, CO, USA) at 38.5°C in a humidified environment with 5% CO_2 for 90 min. The oocytes were rinsed thrice in BSA-PVA-PBS for 5 min each and thereafter mounted on glass slides with a mounting solution (Vectashield; Vector Laboratories, Burlingame, CA, USA). Images of TZPs were captured utilizing a confocal microscope (LSM 880; Zeiss, Oberkochen, Germany). The image was converted to black and white applying ImageJ Software for ease. The quantity of observable actin filaments that could be seen passing through the zona pellucida and extending onto the oocyte surface was quantified using ImageJ software. Twenty oocytes from each treatment group were utilized to enumerate the TZPs.

2.11 | Mitochondrial membrane potential

To assess the mitochondrial membrane potential, in vitro grown oocytes at day 5 were denuded and washed with PVP-PBS in control and melatonin supplemented group (10^{-9} M). After washing, oocytes were incubated in the MT1 working solution (1:1000 dilution with MEM) for 30 min in the incubator. To remove excess dye, oocytes were washed with PBS. After washing twice with PVP-PBS, the oocytes were put in a glass-bottomed dish with imaging buffer solution. The fluorescence emission was observed and recorded using a microscope (EVOS® FL; Thermo Fisher Scientific).

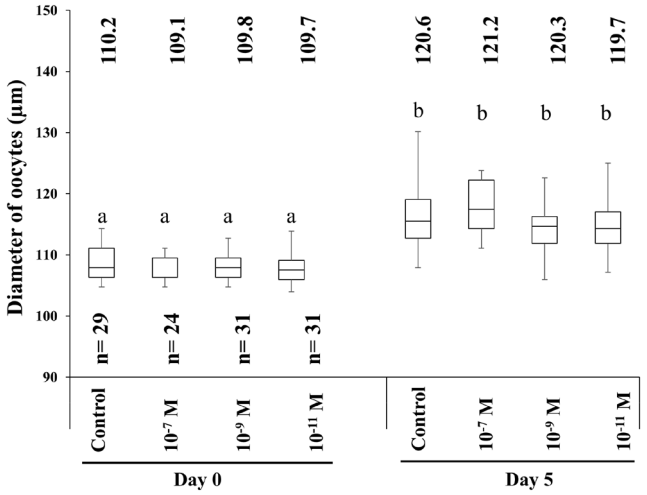

FIGURE 1 Effects of melatonin on the IVG of bovine oocytes. The quantity of oocytes is specified at the bottom of each treatment, while the average diameters are denoted at the top. Boxes exhibiting same patterns represent whisker plots of oocyte diameters prior to (day 0) and subsequent to (day 5) growth culture. The concentrations (M) of melatonin are indicated beneath the graph. Distinct letters in the boxes denote significant differences ($p < 0.05$). OGC, oocyte-granulosa cell complex; IVG, in vitro growth.

2.12 | Statistical analysis

Statistical analyses were conducted by applying R Studio (R Foundation for Statistical Computing, Vienna, Austria) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Each experiment

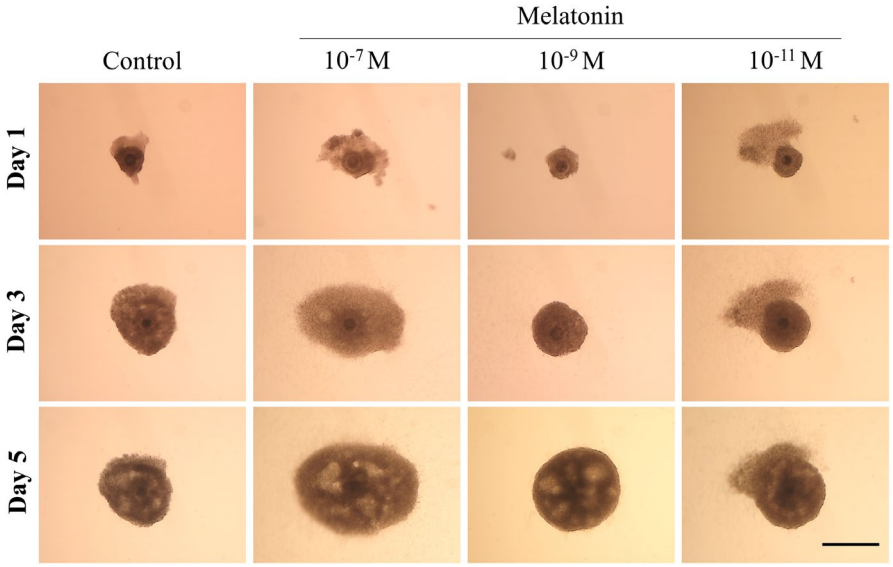

FIGURE 2 Typical morphology of bovine OGCs during IVG culture. OGCs were grown with different concentrations of melatonin (M), and those cultured without melatonin were designated as "control." The days are denoted on the left of the figure. Scale bar denote 500 μm. OGC, oocyte-granulosa cell complex; IVG, in vitro growth.

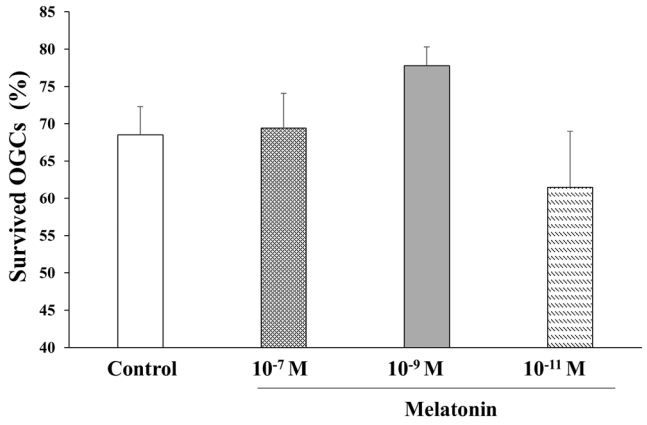

FIGURE 3 Viable oocytes exhibiting optimal cumulus integrity on the fifth day of IVG with different melatonin treatments (M). Data are expressed as the mean ± SEM derived from a minimum of three replicates. OGC, oocyte-granulosa cell complex; IVG, in vitro growth.

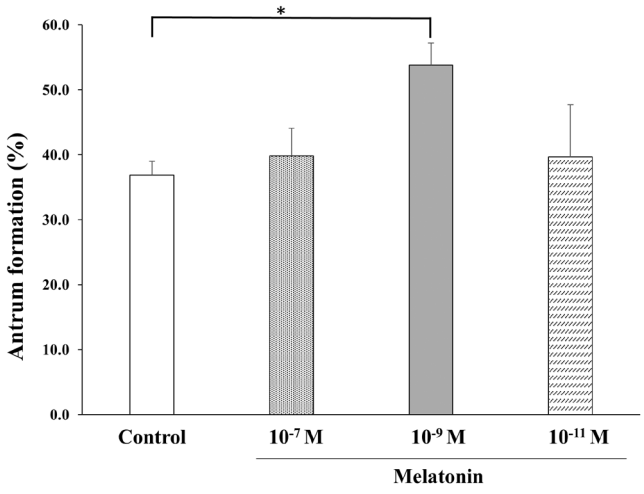

FIGURE 4 Formation of antrum in bovine OGCs on the fifth day during IVG culture supplemented with different concentrations of melatonin (M). Data are expressed as the mean ± SEM derived from a minimum of three replicates. Asterisks denote significant differences (*p <0.05). OGC, oocyte-granulosa cell complex; IVG, in vitro growth.

had a minimum of three replicates unless specified differently. Data are expressed as the mean ± standard error of the mean (SEM). Processing of data, graphical representation, and box-and-whisker plots were produced utilizing Microsoft Excel. The differences between the two groups were analyzed by applying Student's t-test. A one-way analysis of variance was applied on further experimental data, followed by Tukey's HSD test. Statistical significance was determined at p <0.05.

3 | RESULTS

3.1 | Effect of melatonin on oocyte diameter

Oocyte diameters were recorded on day 0 and day 5 of IVG. Figure 1 shows the diameter changes during IVG. The characteristic morphology of in vitro-grown OGCs is displayed in Figure 2.

On day 5, the OGCs were denuded to measure their precise diameter. From day 0 to 5, the diameters accelerated (p < 0.05) in the control group as well as all the treatment groups. Both the melatonin-supplemented and control groups exhibited an increase in oocyte diameter on day 5, comparable to that seen in in vivo-grown oocytes. No significant changes were noticed among the melatonin-treated groups on the fifth day.

3.2 | Effects of melatonin on the survival and antrum formation rate of oocytes

Oocyte survival and antrum formation were evaluated on day 5 of IVG. Oocytes with a partial or complete detachment of cumulus cells

TABLE 2 Effect of melatonin on the subsequent maturation of in vitro-grown oocytes.

Treatment	No. of oocytes	GVBD%	MI%	MII%
Control	41	38.3 ± 5.5	22.4 ± 6.4	39.3 ± 3.8
10^{-7} M	28	51.0 ± 7.7	10.3 ± 6.6	38.6 ± 6.9
10^{-9} M	69	48.4 ± 4.2	5.2 ± 3.3	46.3 ± 2.8
10^{-11} M	42	44.4 ± 3.5	11.1 ± 3.5	44.4 ± 4.6

Note: In vitro-grown OGCs were allowed to undergo subsequent IVM. 10^{-7} M, 10^{-9} M, 10^{-11} M; melatonin concentration. Data are expressed as mean ± SEM.

Abbreviations: IVM, in vitro maturation; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; OGCs, oocyte-granulosa cell complexes.

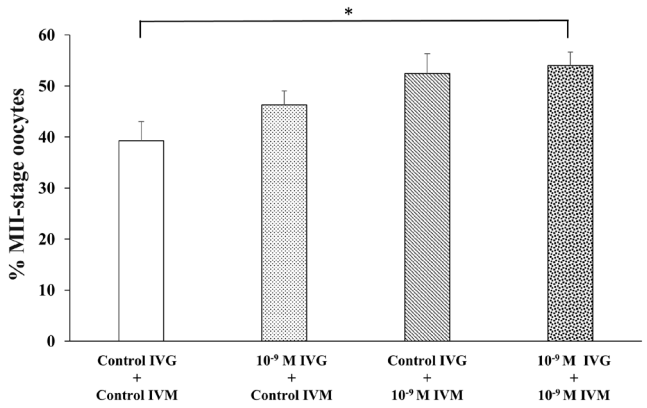

FIGURE 5 Impact of melatonin on the maturation of in vitro-grown oocytes. In the control, only basic growth culture medium was used, and maturation competence was assessed after 5 d of growth. In the 10^{-9} M group, melatonin was supplied to the IVG medium and maturation competence was assessed. Then, melatonin (10^{-9} M) was added only in IVM medium of control IVG grown oocyte. In the $10^{-9} + 10^{-9}$ M group, melatonin was supplied to both the IVG and IVM media. Data are expressed as the mean ± SEM derived from a minimum of three replicates. Asterisks denote significant differences (*p <0.05). IVG, in vitro growth; IVM, in vitro maturation.

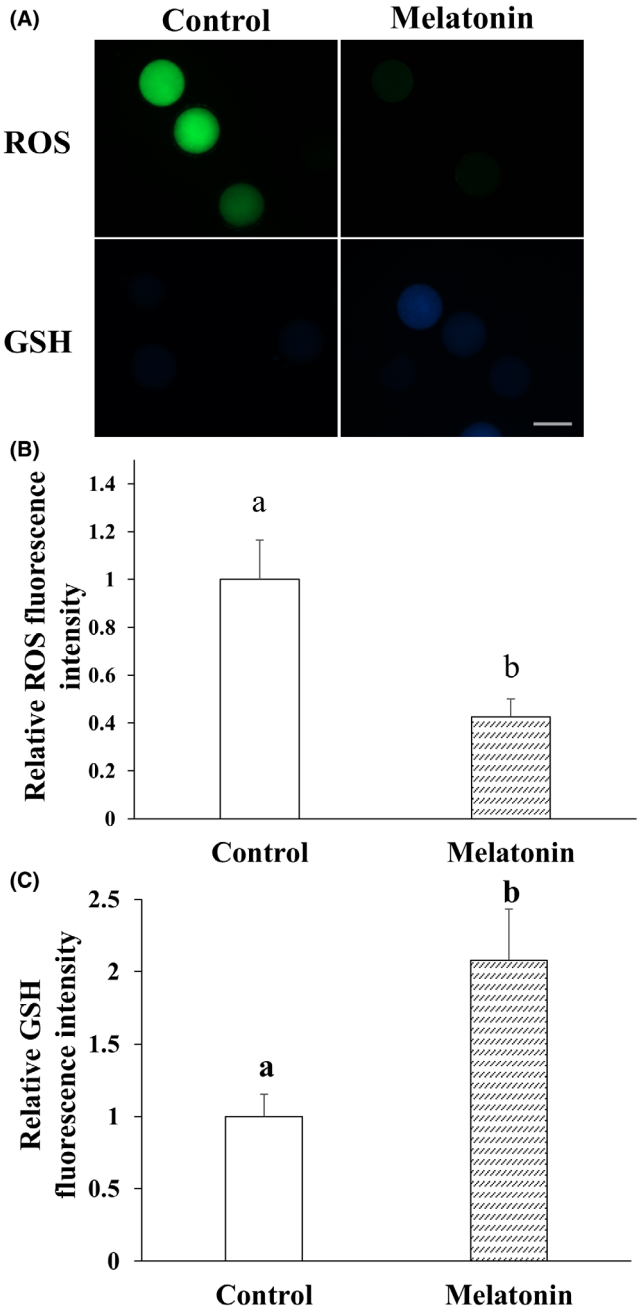

FIGURE 6 Role of melatonin on intracellular ROS levels in oocytes on the fifth day of IVG. Representative image of ROS (green) and GSH (blue) fluorescence in denuded oocytes of the control and melatonin-treated groups (A). The ROS (B) and GSH levels (C) in the oocytes of the control and melatonin-treated (10^{-9} M) groups were detected on day 5 of IVG. Data are expressed as mean ± SEM. The letters "a, b" show significantly different ($p < 0.05$). Scale bar indicates 100 μm. ROS, reactive oxygen species; GSH, reduced glutathione; IVG, in vitro growth.

were discarded, whereas those with well-attached cumulus cells were deemed to have survived and were utilized in further experiments. The surviving oocytes had antrum-like spaces or dome-like structure formed by well attached cumulus and granulosa cells. The standard morphology of oocytes that survive on day 5 is depicted in Figure 2. The survival of OGCs is displayed in Figure 3. No notable changes were seen in the surviving oocytes in the control and other melatonin-supplemented groups.

Figure 4 displays the rate of antrum development in OGCs during IVG. The oocytes were encircled by granulosa and cumulus cells upon collection. Cumulus and granulosa cells underwent proliferation during the culture period, and layered cumulus cells formed dome and antrum-like structures (Figure 2). The antrum was distinctly observable on day 5 (Figure 2). A higher percentage of OGCs showed antrum-like structures in the 10^{-9} M melatonin-treated group compared to the control group ($p < 0.05$), indicating that melatonin (10^{-9} M) promoted antrum formation. Thus, a concentration of 10^{-9} M was considered ideal for the IVG of oocytes, and other experiments on ROS, GSH, gap junctions, TZP, mitochondrial membrane potential, and mRNA expression were performed using 10^{-9} M melatonin.

3.3 | Impact of melatonin treatment on the IVM of bovine oocytes

After 5 d of IVG with different concentrations of melatonin, OGCs were allowed to mature in vitro to assess their subsequent maturation competence. After IVM, chromosomal configuration was identified using aceto-orcein staining. The results indicated that 39.3% of the oocytes reached MII in the control group, whereas 46.3% of the oocytes reached MII in the 10^{-9} M melatonin-treated group, although significant changes were not found in IVM rates from the melatonin-supplemented in vitro-grown oocytes or control (Table 2). Furthermore, the addition of 10^{-9} M melatonin to the IVG and IVM medium resulted in a significant increase in the maturation rate to 54.04% compared to the control group (p <0.05) (Figure 5). Thus, melatonin during IVG may not affect maturation, but melatonin (10^{-9} M) in both IVG and IVM improves the maturation rate.

3.4 | Effects of melatonin on ROS and GSH levels and H_2O_2-induced oxidative stress

OGCs were denuded on day 5 of IVG and intracellular ROS and GSH levels were examined to assess oxidative stress and GSH, respectively, in the control and 10^{-9} M melatonin-treated groups. Figure 6A displays typical images of the oocytes' ROS and GSH levels. The melatonin-administered group had a reduced relative ROS fluorescence intensity compared to the control group (p <0.05) (Figure 6B). Regarding GSH, greater fluorescence intensity was noted in the group treated with melatonin compared to the control (p < 0.05) (Figure 6C). Melatonin supplementation reduced oxidative stress during IVG and increased intracellular glutathione levels. To determine whether melatonin suppresses oxidative stress in OGCs, we added H_2O_2 (100 μM) to the IVG medium to induce oxidative stress, followed by culturing for 2 h with or without melatonin and melatonin alone. Representative photos are displayed in Figure 7A. A higher H_2O_2 fluorescence intensity was observed in the induced oxidative stress group, whereas melatonin significantly suppressed this stress (p < 0.05) (Figure 7B).

3.5 | Melatonin treatment increased the GJC and TZP number

At the end of IVG, the GJC of IVG-grown oocytes and the number of TZPs were checked to assess cumulus-oocyte communication. TZPs are vital structures that maintain the gap junctions based

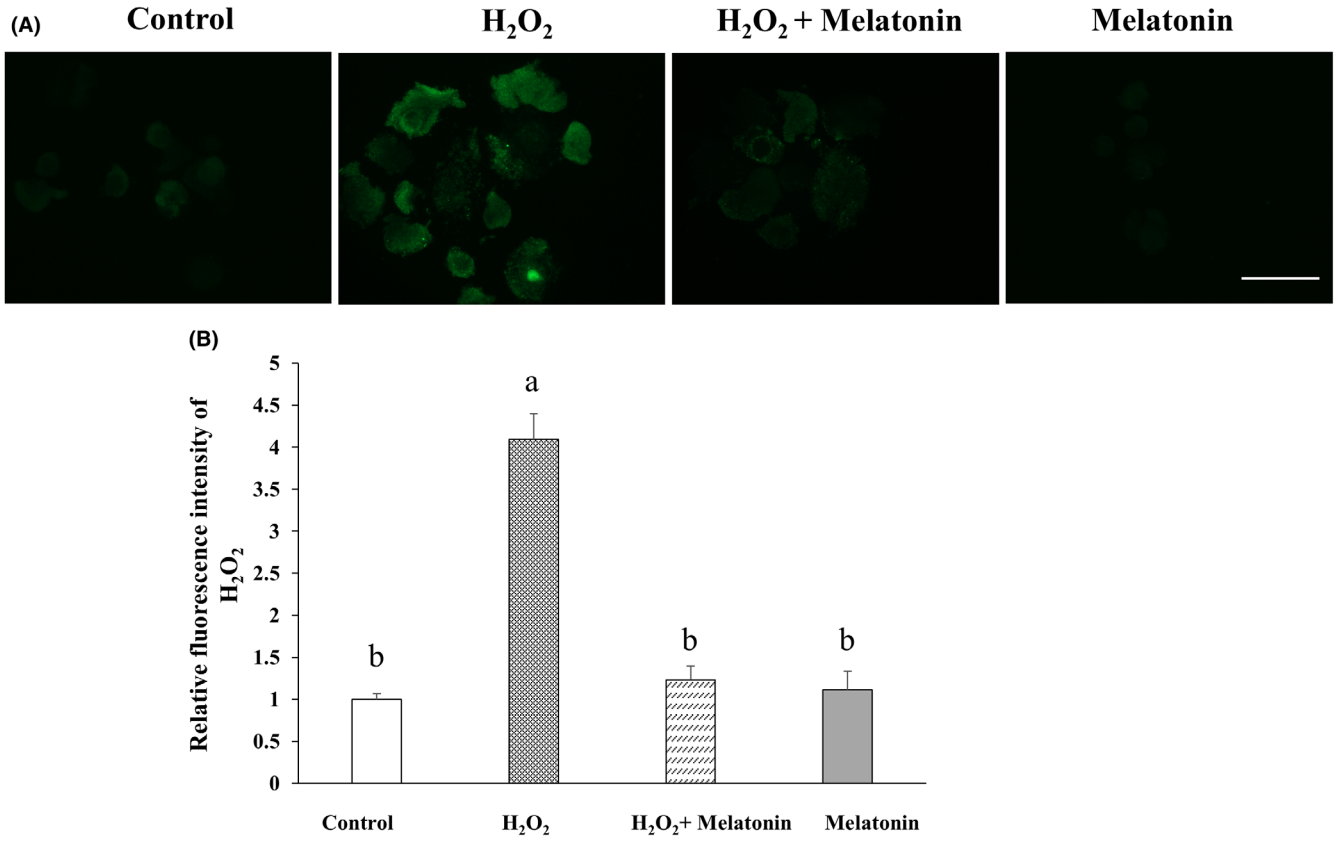

FIGURE 7 Role of melatonin on hydrogen peroxide fluorescence levels in OGCs. Representative fluorescence image of control and H_2O_2-, H_2O_2 with melatonin-, and melatonin-supplemented groups (A). To elucidate the suppressive effect of melatonin on oxidative stress, OGCs were cultured with H_2O_2 (100 μM), H_2O_2 with melatonin (10^{-9} M), or melatonin (10^{-9} M) for 2 h in IVG medium (B). Data are expressed as mean ± SEM. The letters "a, b" show significantly different values (p < 0.05). Scale bar represents 500 μm. OGC, oocyte-granulosa cell complex; IVG, in vitro growth.

on reciprocal interaction between oocytes and cumulus cells. The fluorescence shots were transformed to black and white pictures to make TZPs counting easier. In Figure 8A, typical TZP images are shown. The melatonin-treated group exhibited a greater total number of TZPs compared to the control group (p <0.05) which suggests increased GJC (Figure 8B).

Representative images of open, partially open, and almost closed states of gap junctions during calcein dye transfer from cumulus cells to oocytes are shown in Figure 9A. Analysis of the fluorescence image revealed higher GJC in the melatonin-supplemented group than in the control group (p <0.05) (Figure 9B). TZPs may notably contribute to the formation of gap junctions, as a substantial number of TZPs were found in the melatonin-treated group.

3.6 | Mitochondrial membrane potential

Mitochondrial membrane potential of oocytes was checked on day 5 of IVG in control and melatonin treated group with MT1 dye. Representative images are shown in Figure 10A. After analysis, no significant changes were found between control and melatonin supplemented group (10^{-9} M).

3.7 | Melatonin on ER stress-related gene expression in IVG-derived oocytes

Figure 11 shows the mRNA expression of genes related to ER stress. All genes were downregulated in the melatonin-treated group compared to the control. During IVG, the melatonin-treated group had reduced relative mRNA expression levels of *PERK*, *ATF6*, *ATF4*, *CHOP*, *IRE1*, and *XBP1* (p <0.05) (Figure 10). Consequently, melatonin administration (10^{-9} M) during IVG reduced ER stress-related gene expression, as shown by qRT-PCR analysis.

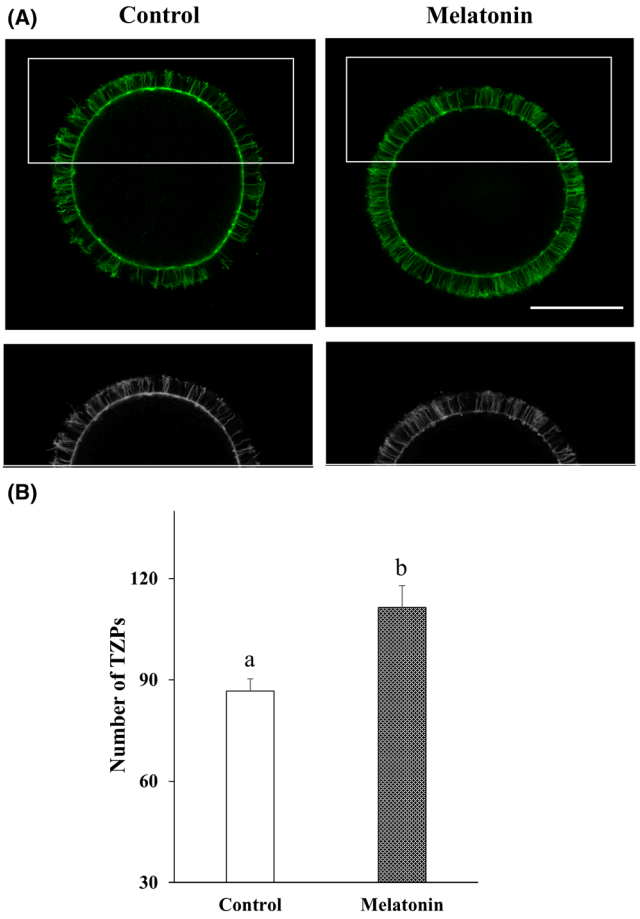

FIGURE 8 Effect of melatonin on the number of TZPs in oocytes during IVG. (A) Representative image of Actin fluorescence staining indicates the location of phalloidin–stained TZPs in oocytes. (B) TZPs were manually counted from the fluorescence image using ImageJ software. To highlight TZPs, the color was inverted to black and white. Data are stated as mean ± SEM. Each group had a total of 20 oocytes tested across three replicates. Distinct letters indicate significantly different values (p < 0.05). Scale bar represents 50 μm. TZP, transzonal projection; IVG, in vitro growth.

4 | DISCUSSION

Oocyte diameter is a predictor of IVM competency. Bovine oocytes with small diameters (105 to 115 μm or less) from early antral follicles are less capable of maturing in vitro compared to fully grown oocytes (≥120 μm) (Figure S1). Using secondary and early antral follicles to their full potential, IVG culture of oocytes offers a fresh supply of developed oocytes and embryos following IVM and in vitro fertilization.[38] The IVG protocol is in the progressive stage in terms of maintaining the quality of oocytes while improving developmental competences using different supplements.[30,39,40] ER stress affects bovine oocyte maturation and embryo yield by accelerating oxidative stress in vitro.[32,41] The present study emphasizes that suppressing the stresses using melatonin improves oocyte quality during IVG.

We queried the growth, survival, antrum formation, and maturation of bovine oocytes in vitro. In addition, we examined the relation of GJC with TZP number, intracellular ROS and GSH levels, and mRNA expression of ER stress-associated genes. Melatonin improved antrum formation in oocytes grown in vitro. When added to the IVM medium, it accelerated the maturation rate relative to the control. The melatonin-supplemented group showed high GJC, increased number of TZPs, heightened intracellular glutathione levels, and reduced ROS levels compared with those in the control group.

Growing granulosa cells produce the steroid hormones 17β-estradiol and androstenedione, which govern the IVG of oocytes by protecting the physical connections of granulosa cells and oocytes throughout IVG.[42] Through gap junctions, granulosa cells ensure the exchange of necessary substances that are pivotal for the growth of oocytes.[38] In this study, IVG culture media were supplemented with steroid hormones, and the oocyte diameter increased on day 5 compared with that on day 0 in all groups. However, the significance of melatonin in increasing the diameter compared with that in the control was not found in the present study.

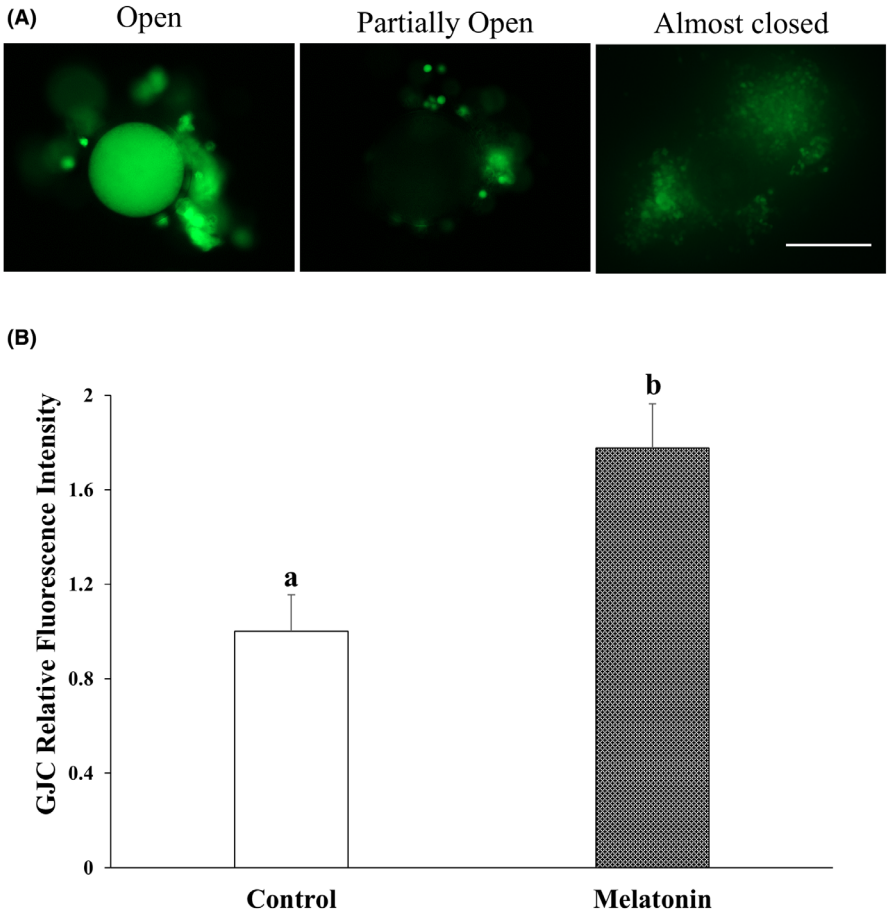

FIGURE 9 Impact of melatonin on cumulus-oocyte GJC during IVG of OGCs. Based on the calcein dye transfer levels from cumulus cells to oocytes, image representation of open, partially open, or almost close gap junction of oocytes (A). The relative fluorescence intensity of GJC quantified using ImageJ (B). Values are stated as mean ± SEM. Each group had a total of 25 oocytes from three replicates tested. Significantly different values are shown by distinct letters ($p < 0.05$). Scale bar represents 100 μm. GJC, gap junction communication; IVG, in vitro growth; OGC, oocyte-granulosa cell complex.

The antrum is a fluid-filled space in the granulosa cell layers formed in mammalian follicles[38] that plays an important role in follicular development. Antrum-like structures have been observed by many researchers in several species during follicle culture and IVG of OGCs.[43–45] Shen et al. reported that oocyte-secreted factors (OSFs) might induce antrum formation during IVG culture.[46] OSFs such as GDF9 and BMP15 are crucial for antrum-like structure formation during IVG, while combined supplementation to GC or OXC induces the formation of more antrum-like structures.[47] Melatonin has been reported to increase *GDF9* and *BMP15* levels in follicle-enclosed oocytes in the dark phase.[48] Melatonin supplementation in mice increased *GDF9* and *BMP15* expression levels in oocytes, which affect the TZPs and GJC.[49] Melatonin may affect the levels of OSFs such as GDF9 and BMP15 in vitro, which could have led to an increase in antrum development in the current investigation.

Melatonin supplementation enhances the maturation competence of bovine and porcine oocytes.[12,50] Heat-stressed porcine oocytes showed increased first polar body extrusion in IVM with exogenous melatonin supplementation.[51] Melatonin upregulated genes associated with oocyte maturation and cumulus cell expansion.[12] The current study indicates that melatonin supplementation during the IVG and IVM (10^{-9} M) improved the maturation rate significantly compared with the control. High concentration did not improve the subsequent maturation rate. Previous report of melatonin on IVM of bovine oocytes revealed that melatonin work in a dose dependant manner and higher concentration reduce the developmental potential which support the current study.[12] The antioxidant and antiapoptotic mechanisms of melatonin protect oocytes from oxidative stress and may improve cytoplasmic maturation, further affecting nuclear maturation.

In the current investigation, melatonin treatment escalated the TZP number and GJC in oocytes cultured in vitro. Originating from cumulus granulosa cells, TZPs maintain communication by linking directly to the oocytes, and at the tip of the TZPs, granulosa cells form the heterologous gap junction.[52,53] Intercellular channels known as gap junctions allow ions, nutrients, mRNA, and small molecules (<1 kDa) to move directly across adjacent cells.[33,54–56] Granulosa cell development and TZP generation are regulated by OSFs.[57] Besides their function in oocyte-cumulus communication, TZPs regulate the oocyte maturation process.[58] In growing oocytes, TZPs are increased in number and contribute to growth. The number of TZPs significantly decreased during IVG culture, and this decrease was prevented by treatment with 17β-estradiol for bovine oocytes[42] and with FSH for porcine oocytes.[59] Melatonin-supplemented oocytes increased the expression of gap junction protein alpha 4, which may also contribute to the enhanced GJC and TZPs.[60] It has been reported that *ERK* activation occurs during oxidative stress in somatic cells.[61] Other findings showed that inhibiting ERK activity blocked the TZP retraction.[62] Melatonin may minimize spontaneous *ERK* activation by reducing oxidative stress, thereby preventing TZP retraction and improving cumulus-oocyte GJC.

ER and oxidative stress escalate one another in regenerative feedback that disrupts cellular processes and triggers proapoptotic signals.[5] Overproduction of ROS damages the oocytes' ability to develop in vitro by impairing protein function and cell structure through intracytoplasmic oxidative damage, which in turn induces cell death.[60,63,64] Intracellular GSH acts as an antioxidant, shielding OGCs from oxidative injury and playing a role in cytoplasmic maturation, meiotic spindle formation, and the reduction of oxidative stress.[65,66] Considering these findings, the expression levels of ROS, GSH, and ER stress-associated genes (*PERK*, *ATF6*, *IRE1*, *ATF4*, *XBP1*, *CHOP*, and *GRP78*) were evaluated in in vitro-grown oocytes in the present study. Reduced ROS levels, downregulated mRNA expression of ER stress-associated genes, and high intracellular glutathione levels were observed in the melatonin-treated group but no significant changes noticed of mitochondrial membrane potential in control and melatonin treated group. Several investigations on bovine, mouse, and porcine oocytes have reported that melatonin suppresses oxidative stress by reducing intracellular ROS levels with an increasing level of intracellular GSH in vitro.[22,23,49] Melatonin may regulate SIRT1 to reduce oxidative stress in vitrified-warmed mouse oocytes as well as promote development.[67] Melatonin suppresses ER stress during the IVM of porcine oocytes.[68,69] These reports support our results on the antioxidant and ER stress attenuation properties of melatonin during the IVG of bovine oocytes. In this investigation, only mRNA expression of ER stress associated genes were checked. Further study is needed to check the protein expression level of concerned genes for better understanding.

In conclusion, melatonin increases antrum formation and granulosa cell proliferation. Cumulus-oocyte GJC through TZPs increased in the melatonin-supplemented group. ROS levels are decreased in oocytes and granulosa cells, indicating a reduction in oxidative stress and an increase in the intacellular glutathione levels in oocytes. ER stress attenuation was detected in the melatonin-treated

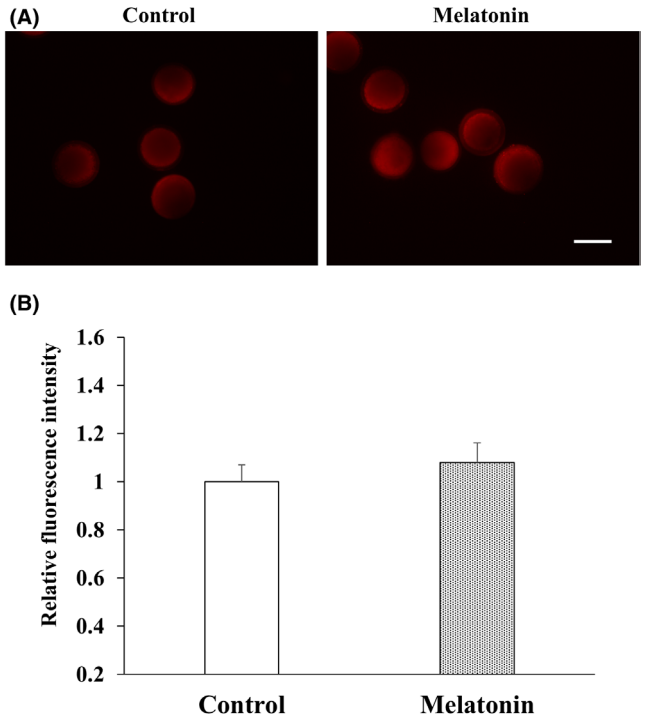

FIGURE 10 Effect of melatonin on mitochondrial membrane potential of IVG grown oocytes. Representative image of in vitro grown oocytes with or without melatonin (10^{-9} M) stained with MT1 to check the mitochondrial membrane potential (Figure A). Relative fluorescence intensity is shown in figure B. All experiments were performed in at least 3 replications. Scale bar represents 100 μm.

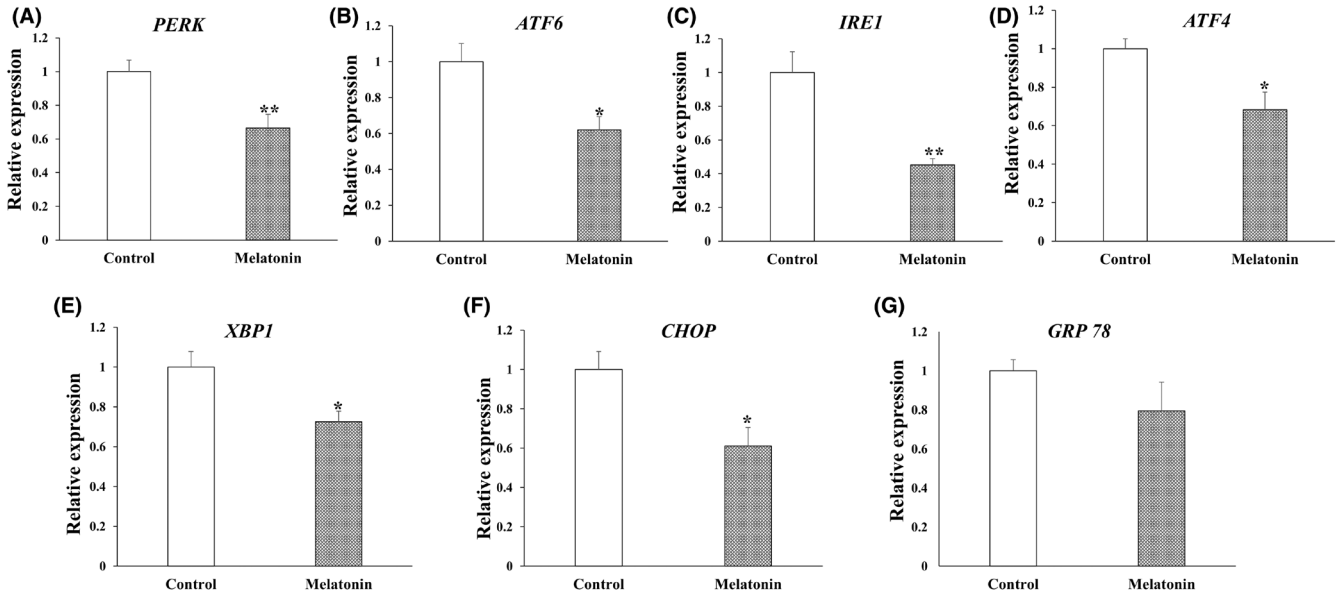

FIGURE 11 Effect of melatonin on the relative expression of ER stress-associated genes (A) *PERK*, (B) *ATF6*, (C) *IRE1*, (D) *ATF4*, (E) *XBP1*, (F) *CHOP*, and (G) *GRP78* in OGCs of the control and melatonin-treated (10^{-9} M) groups on the fifth day of IVG determined using qRT-PCR from three replicates. Values are stated as mean ± SEM. Asterisks marked significant differences (*p <0.05; **p <0.01). ER, endoplasmic reticulum; OGC, oocyte-granulosa cell complex; IVG, in vitro growth.

group through gene expression analysis of ER stress-associated genes using qRT-PCR. The maturation rate improved when melatonin was supplied to the IVM medium. IVG supplementation of melatonin might improve oocytes quality and reduce ER stress but this was not reflected in subsequent maturation rate. Further study is required to explore the melatonin effect on in vitro embryo production from IVG grown oocytes.

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CONFLICT OF INTEREST STATEMENT

The authors disclose no conflicts of interest that may compromise the objectivity of this study.

ETHICS STATEMENT

This article does not include any research involving human or animal subjects conducted by any of the authors.

ORCID

Ken-ichi Yamanaka https://orcid.org/0000-0002-2133-8209

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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