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Melatonin protects the integrity of granulosa cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice

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Abstract

Melatonin protects luteinized granulosa cells (GCs) from oxidative stress in the follicle during ovulation. However, it is unclear in which cellular components (e.g., nuclei, mitochondria, or plasma membranes) melatonin works as an antioxidant. GCs from immature (3 wks) ICR mice were incubated with hydrogen peroxide (H2O2; 0.01, 0.1, 1, 10 mM) in the presence or absence of melatonin (100 μg/ml) for 2 h. DNA damage was assessed by fluorescence-based immunocytochemistry using specific antibodies for 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative guanine base damage in DNA, and for histone H2AX phosphorylation (γH2AX), a marker of double-strand breaks of DNA. Mitochondrial function was assessed by the fluorescence intensity of MitoTracker Red probes, which diffuse across the membrane and accumulate in mitochondria with active membrane potentials. Lipid peroxidation of plasma membranes was analyzed by measuring hexanoyl-lysine (HEL), a oxidative stress marker for lipid peroxidation. Apoptosis of GCs was assessed by nuclear fragmentation using DAPI staining, and apoptotic activities were evaluated by caspase-3/7 activities. H2O2 treatment significantly increased the fluorescence intensities of 8-OHdG and γH2AX, reduced the intensity of MitoTracker Red in the mitochondria, increased HEL concentrations in GCs, and enhanced the number of apoptotic cells and caspase-3/7 activities. All these changes were significantly decreased by melatonin treatment. Melatonin reduced oxidative stress-induced DNA damage, mitochondrial dysfunction, lipid peroxidation, and apoptosis in GCs, suggesting that melatonin protects GCs by reducing oxidative stress of cellular components including nuclei, mitochondria, and plasma membranes. Melatonin helps to maintain the integrity of GCs as an antioxidant in the preovulatory follicle.

Keywords: Granulosa cells, Melatonin, Oxidative stress, Reactive oxygen species

Introduction

Granulosa cells are induced to differentiate into luteal cells by a surge of ovulatory luteinizing hormone (LH). The ovulatory LH surge induces the expression of steroidogenic acute regulatory (StAR) protein, a rate-limiting enzyme for progesterone synthesis [1-3]. Progesterone produced by granulosa cells undergoing luteinization is necessary for ovulation and subsequent corpus luteum formation. On the other hand, during ovulation after the LH surge, reactive oxygen species (ROS) are locally produced by macrophages, neutrophils and vascular endothelial cells within the follicle [4], suggesting that granulosa cells are exposed to elevated levels of ROS. ROS play a physiological role in ovulation, e.g., in follicle rupture, while an excessive amount of ROS can damage both the ovum and the granulosa cells undergoing luteinization.

Reactive species damage cellular components including nuclei, mitochondria and plasma membranes, resulting malfunctioning DNA, loss of membrane integrity and mitochondrial dysfunction; the latter in particular is often related to apoptosis [5-8]. Importantly, antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase, and non-enzymatic antioxidants such as melatonin, vitamin E, vitamin C, glutathione, uric acid and albumin are present in the follicles [9-11]. The balance between ROS and the antioxidants within the follicle seems to be critical to the integrity and function of granulosa cells undergoing luteinization during ovulation.

Melatonin and its metabolites are powerful free radical scavengers and broad-spectrum antioxidants [12-14]. Interestingly, melatonin is present in high concentrations in the preovulatory follicle [15-19], and the concentration of melatonin in follicular fluids increases with increasing follicle size [15, 16]. Because of its small size and highly lipophilic properties [12, 20], melatonin passes through all cell membranes and easily reaches cellular components including nuclei, mitochondria, and plasma membranes, where it seems to accumulate in high concentrations [21-23]. Melatonin prevents DNA damage [24, 25] and lipid peroxidation of plasma membranes [26, 27]. In particular, melatonin preserves optimal mitochondrial function and homeostasis by reducing and

preventing oxidative stress, thereby curtailing subsequent apoptotic events and cell death [12, 28, 29]. We recently reported that hydrogen peroxide (H_2O_2) inhibited progesterone production by human luteinized granulosa cells and that melatonin abolished the inhibitory effect of H_2O_2 [30]. However, it is unclear on which cellular components melatonin works as an antioxidant to protect granulosa cells. Therefore, this study was conducted to investigate whether melatonin reduces oxidative stress in granulosal cellular components including in the nuclei, mitochondria, and plasma membranes.

Materials and methods

Collection and culture of granulosa cells

The experimental protocol was approved by the Committee for Ethics on Animal Experimentation, and performed under the Guidelines for Animal Experiments at Yamaguchi University Graduate School of Medicine in accordance with Law No. 105 and Notification No. 6 of the Japanese Government. Immature (3 wks) ICR mice (Japan SLC Inc., Hamamatsu, Japan) were housed in a controlled room with a 14:10 light:dark photoperiod and free access to standard mouse chow and water. All mice received a subcutaneous injection of 20 units of pregnant mare serum gonadotropin (PMSG, G4877; Sigma-Aldrich, St. Louis, MO, USA) to stimulate the development of multiple follicles. All mice were laparotomized under deep ether anesthesia 48 h after the PMSG injection; the ovaries were quickly removed for the following experiments, and the mice were euthanized by exsanguinations. The ovaries were transferred to alpha Modified Eagle Minimum Essential Medium (αMEM) without phenol red (M4655, Sigma-Aldrich) supplemented with penicillin-streptomycin (15070-063, Invitrogen, Carlsbad, CA, USA). Granulosa cells were collected by puncturing mature preovulatory follicles with a 26-gauge needle under a dissecting microscope. Granulosa cells were isolated, centrifuged at 800 x g, washed in PBS (Wako Pure Chemical Industries, Osaka, Japan) twice, and used for cell culture. The cells were preincubated at a density of 2.5 x 10^4 cells/well in 100 μl of αMEM for 30 min, and then incubated with H2O2 (8104215, 0.01, 0.1, 1, and 10 mM; Wako Pure Chemical Industries) in the presence or absence of melatonin (M5250, 100 μg/ml; Sigma-Aldrich) for 2 h. After incubation, cells were used for evaluation of DNA damages, mitochondrial function, lipid peroxidation, and apoptosis as described below.

DNA damage

DNA damage was assessed by fluorescence-based immunocytochemistry using specific antibodies for 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative guanine base damage of

105 DNA, and for histone H2AX phosphorylation (γH2AX), a marker of double-strand breaks of DNA.
106 Granulosa cells were fixed with 4% paraformaldehyde (L3N8367, Nacalai Tesque, Kyoto, Japan) for
107 15 min, washed three times in PBS, and incubated in 0.5% Triton X-100 (T8787, Sigma-Aldrich) in
108 PBS for 15 min. Then, the cells were incubated with primary antibodies against either 8-OHdG
109 (mouse monoclonal IgG, sc-66036, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA)
110 at room temperature for 2 h, and they were then incubated with a secondary antibody (Alexa Fluor
111 488-labeled goat anti mouse IgG, A-11001, 1:200 dilution; Invitrogen) at room temperature for 40 min.
112 The cells were also incubated with the antibody against γH2AX (Alexa Fluor 488-labeled rabbit
113 monoclonal IgG, 9719S, 1:100 dilution; Cell Signaling Technology, Danvers, MA, USA) at room
114 temperature for 2 h. To counterstain the DNA material, granulosa cells were mounted onto slides
115 and counterstained with 50 μg/ml 4',6'-diamidino-2-phenylondole (DAPI, 340-07971; Wako Pure
116 Chemical Industries) in Vectashield mounting medium (H-1200, Vector Laboratories, Burlingame,
117 CA, USA) under a cover slip. Fluorescence of 8-OHdG and γH2AX in the nuclei was detected and
118 imaged under a confocal laser scanning microscope (Zeiss LSM 510 META; Carl Zeiss, Jena,
119 Germany) utilizing a 488 nm excitation. Fluorescence images were captured by ZEN imaging
120 software (ZEN 2008; Carl Zeiss), and the fluorescence intensity from each cell was quantified using
121 the CellProfiler and ImageJ software (National Institutes of Health, Bethesda, MD, USA). The
122 fluorescence intensities of 8-OHdG and γH2AX were measured in at least 200 cells per treatment
123 group, and the mean fluorescence intensity was used as the level of DNA damage.
124
125 *Mitochondrial function*
126 Mitochondrial function was evaluated by the mitochondrial membrane potential, which was
127 quantified by using mitochondrial-targeted fluorescent probes (MitoTracker Red, M7512, Invitrogen).
128 MitoTracker Red diffuses across cell membranes and accumulates in mitochondria with active
129 membrane potentials. Granulosa cells were incubated with MitoTracker Red (100 nM) at room

temperature for 15 min. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min. Then, the cells were washed three times with PBS and mounted on glass slides. Fluorescence was detected and imaged under a confocal laser scanning microscope (Zeiss LSM 510 META) utilizing 543 nm excitation. Fluorescence images were captured by the ZEN software, and the fluorescence intensity and area of MitoTracker Red staining in at least 200 granulosa cells was quantified by the ImageJ software. A reduction in the intensity and area of MitoTracker Red staining is an indicative of reduced mitochondrial membrane potentials.

Hexanoyl-lysine (HEL) *assay*

Lipid peroxidation of cell membranes was analyzed by measuring HEL, a stable oxidative stress marker for lipid peroxidation. HEL levels in cells were measured with a competitive enzyme-linked immunosorbent assay (ELISA) kit (KHL-700, Japan Institute for the Control of Aging, Nikken SEIL, Shizuoka, Japan) as reported previously [17]. After incubation, granulosa cells were resuspended in 100 μl αMEM, lysed by sonication and used for the HEL assay. The assay procedures were performed according to the manufacturer's recommendations. Lipid peroxidation of cell membranes was determined as the overall HEL level per well with $2.5x10^4$ granulosa cells. The minimal detectable concentration of HEL was estimated to be 2 nmol/l.

Apoptosis

Apoptosis of granulosa cells was assessed by morphological changes of nuclei and caspase-3/7 activities. Apoptotic morphological changes of nuclei were evaluated by DAPI staining. Granulosa cells were fixed with 4% paraformaldehyde for 15 min. Then, the cells were washed three times in PBS, mounted on glass slides, and stained with 50 μg/ml DAPI in Vectashield mounting medium under a cover slip. Fluorescence was detected and imaged under a confocal laser scanning microscope (Zeiss LSM 510 META). Apoptotic cells were identified by condensation and

fragmentation of the nuclei, and apoptosis was quantified by calculating the percentage of apoptotic nuclei in a total of 200 nuclei in each treatment group.

Caspase-3/7 activities were determined by a Caspase-Glo™ 3/7 Assay (G8091, Promega, Mannheim, Germany) according to the manufacturer's protocol. Briefly, granulosa cells were cultured on a 96-well plate with $2.5x10^4$ cells in 100 µl medium/well, and 100 µl of Caspase-Glo® reagent was added to each well and incubated for 2 h at room temperature. The luminescence of each well was measured by luminometer (Berthold Micro Lumat LB96P; Berthold Technologies, Bad Wildbad, Germany) with an excitation wavelength of 499 nm and an emission wavelength of 521 nm. Apoptotic activities were quantified as the level of fluorescence emitted from Caspase-Glo® reagent bound to caspase-3/7. Caspase-3/7 activities were determined as the overall activities per well with $2.5x10^4$ granulosa cells and shown as relative fluorescence units (RFU).

Statistical analysis

All experiments were performed with three independent incubations. Statistical analysis was carried out using the computer program SPSS for Windows13.0. The Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction analysis were used. A value of $P < 0.05$ was considered significant.

Results

DNA damage in granulosa cells was assessed by fluorescence-based immunocytochemistry using specific antibodies for 8-OHdG (an indicator of oxidative guanine base damage of DNA) and γH2AX (a marker of double-strand breaks of DNA). The fluorescence intensity of 8-OHdG was dose-dependently increased by H2O2 treatment (Fig. 1A-1D), and the increase in 8-OHdG intensities induced by H2O2 was completely blocked by melatonin treatment (Fig. 1E-1I). The fluorescence intensity of γH2AX was also dose-dependently increased by H2O2 treatment (Fig. 2A-2D), and the increase in γH2AX intensities induced by H2O2 was completely blocked by melatonin treatment (Fig. 2E-2I).

Mitochondrial function of granulosa cells was assessed by the fluorescence intensities of MitoTracker Red probes, which diffuse across the membrane and accumulate in mitochondria with active membrane potentials. The intensity of MitoTracker Red was significantly decreased by 10 mM of H2O2 (Fig. 3A-3E). The decrease in MitoTracker Red intensities caused by H2O2 was significantly reversed by melatonin treatment (Fig. 3F-3J).

Lipid peroxidation of plasma membranes in granulosa cells was evaluated by measuring HEL, a stable oxidative stress marker for lipid peroxidation. The HEL concentrations were significantly increased by 10 mM of H2O2 (Fig. 4A), and the increase was significantly blocked by melatonin treatment (Fig. 4B).

Apoptosis of granulosa cells was assessed by nuclear fragmentation using DAPI staining and by the caspase-3/7 activities. The percentage of apoptotic cells was dose-dependently increased by H2O2 (Fig. 5A), and the increase in numbers of apoptotic cells induced by H2O2 was completely blocked by melatonin treatment (Fig. 5B). Caspase-3/7 activities in granulosa cells were significantly increased by 10 mM of H2O2 (Fig. 6A), and the increase was significantly decreased by melatonin treatment (Fig. 6B).

198 **Discussion**

199 The present study showed that oxidative stress damages nuclei, mitochondria and plasma
200 membranes in granulosa cells, resulting in DNA damage, mitochondrial dysfunction, and lipid
201 peroxidation of plasma membranes, which likely cause disruption of cellular integrity and apoptosis.
202 Furthermore, our results clearly showed that melatonin protects the integrity of granulosa cells by
203 reducing oxidative stress in each of these cellular components.

204 Various indicators of DNA damage have been reported; these include, oxidative base damage,
205 telomere shortening, chromosome fragmentation, single-strand breaks and double-strand breaks. The
206 double-strand breaks are the most lethal forms of DNA damages because they cause cellular
207 senescence and apoptosis [31-33]. In the present study, DNA damage by oxidative stress was
208 evaluated by γH2AX (a sensitive marker of double-strand breaks) [33] in addition to 8-OHdG (an
209 indicator of oxidative guanine base damage). The present study showed that melatonin blocks both
210 oxidative stress-induced guanine base damages and double-strand breaks in granulosa cells. This is
211 consistent with previous reports that melatonin reduces the DNA damage as assessed by γH2AX in rat
212 germ cells [34] and the rat brain [35].

213 The mitochondrial membranes are important sites for steroidogenesis in granulosa cells.
214 Cholesterol, a substrate of steroid hormones, is transferred from the outer to the inner mitochondrial
215 membrane by StAR protein [36, 37], which is in turn metabolized to pregnenolone by the cytochrome
216 P450 cholesterol side-chain cleavage enzyme (P450scc). Damage to mitochondrial membranes by
217 oxidative stress impairs steroidogenesis in granulosa cells. In fact, oxidative stress has been reported
218 to inhibit steroidogenic enzymes and a mitochondrial carrier protein (StAR protein) involved in
219 cholesterol transport into mitochondria of luteal cells [38]. The present study showed that oxidative
220 stress damages mitochondrial function of granulosa cells and that melatonin reduces the oxidative
221 stress in the mitochondria, suggesting that melatonin protects granulosa cells by reducing the
222 mitochondrial membrane damage caused by oxidative stress. These findings support the previous

223 reports that melatonin preserves mitochondrial function by reducing electron leakage and protecting
224 the mitochondrial membrane [39, 40] and that melatonin increases respiratory chain complex I and IV
225 activities and ATP synthesis [41].

226 Oxidative stress-induced damage to plasma membranes was evaluated by lipid peroxidation. A
227 number of studies have reported that oxidative stress inhibits progesterone production by luteal cells
228 through lipid peroxidation of the plasma membrane [38, 42]. Our previous study also showed that
229 H2O2 inhibits progesterone production by human luteinized granulosa cells and that melatonin
230 abolished the inhibitory effect of H2O2 on progesterone production [30]. Furthermore, it has been
231 reported that lipid peroxidation of the plasma membranes of luteal cells is involved in corpus luteum
232 regression through the disruption of cellular integrity [43, 44]. Taken together, the present results
233 document that melatonin prevents oxidative stress-induced lipid peroxidation of plasma membranes in
234 granulosa cells, as in other cells.

235 The primary function of mitochondria is to generate ATP through the five-complex electron
236 transport chain in the mitochondrial membrane. Mitochondrial damage by oxidative stress is
237 commonly related to cell death. Oxidative stress induces the mitochondrial membrane to release
238 cytochrome c, which activates caspase-9 activity and triggers the downstream caspase cascade
239 including the activation of caspase-3 [45]. Caspases are central mediators of apoptosis [46]; in
240 particular, caspase-3 activation is a major contributor to apoptotic processes [47]. The present study
241 showed that oxidative stress increased the caspase-3/7 activities and the percentage of apoptotic
242 granulosa cells; moreover, the results showed that the apoptotic effects of oxidative stress were
243 blocked by melatonin treatment. Thus, melatonin likely prevented apoptosis of granulosa cells by
244 reducing oxidative stress. These data are consistent with recent reports that melatonin prevents
245 apoptosis by regulating caspase-3 and Bax/BCL-2 in the gastric mucosa [48], leukocytes [49], and
246 bone marrow mesenchymal stem cells [50].
247

Although melatonin directly scavenges free radicals, recent reports showed that melatonin receptors are present in granulosa cells [51, 52]. However, the role of receptor-mediated actions of melatonin in ovarian function is unclear, and little information is available concerning how melatonin receptors change during estrous cycle. It has been reported that melatonin increases the expression of other antioxidant enzymes such as SOD and glutathione peroxidase through melatonin receptors [53, 54]. In this study, the antioxidant effect was found 2 h after melatonin treatment, suggesting the direct antioxidant action of melatonin, but not the receptor-mediated action. However, it would be interesting to investigate whether melatonin works as an antioxidant through its receptors, for example, by upregulating the expression of antioxidant enzymes, in a future study.

H2O2 caused significant DNA damage at the concentration of 10 μM, while concentrations of H2O2 higher than 10 mM were necessary to cause mitochondrial damages, lipid peroxidation and apoptosis with increased caspase 3/7 activities. The difference in the effective concentrations of H2O2 may be due to the difference in sensitivities to ROS among cellular components of granulosa cells. DNA has high sensitivity to ROS, but it is rapidly repaired. In contrast, mitochondria is relatively resistant to ROS because it is always exposed to ROS that it produces. Therefore, DNA is damaged by relatively low H2O2 concentrations, and higher concentrations of H2O2 are necessary to cause mitochondrial damages and the subsequent apoptotic events.

The melatonin concentration used in this study was high compared with the physiological concentrations in the follicle [15]. After melatonin reacts with ROS, the melatonin metabolites are produced and accumulated in vivo. Interestingly, the melatonin metabolites also work as antioxidants, resulting in melatonin and melatonin metabolites working together as powerful antioxidants in vivo. Therefore, a high concentration of melatonin was used in this study to well reflect the in vivo condition.

The present study showed that melatonin reduces the oxidative stress-induced DNA damage, mitochondrial dysfunction, lipid peroxidation, and apoptosis of granulosa cells, showing that

melatonin protects these cells by reducing free radical damage of cellular components including nuclei, mitochondria, and plasma membranes. We previously reported that melatonin is present in high concentrations in the preovulatory follicle [15, 16]. Collectively, these results suggest that melatonin helps to maintain the integrity of granulosa cells in the follicle as an antioxidant. In addition, we recently found that melatonin protects oocytes from oxidative stress in the follicle during ovulation [15-19, 55]. Thus, melatonin, acting as an antioxidant, contributes to not only oocyte maturation but also luteinization of granulosa cells in the follicle during ovulation.

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References

1. **Hickey GJ, Chen SA, Besman MJ, Shively JE, Hall PF, Gaddy-Kurten D, Richards JS.** Hormonal regulation, tissue distribution, and content of aromatase cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis. *Endocrinology* 1988; **122**:1426-1436.
2. **Lee L, Asada H, Kizuka F, Tamura I, Maekawa R, Taketani T, Sato S, Yamagata Y, Tamura H, Sugino N.** Changes in histone modification and DNA methylation of the StAR and Cyp19a1 promoter regions in granulosa cells undergoing luteinization during ovulation in rats. *Endocrinology* 2013; **154**:458-470.
3. **Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM, Orly J.** Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* 1998; **139**:303-315.
4. **Brannstrom M, Norman RJ.** Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Hum Reprod* 1993; **8**:1762-1775.
5. **Halliwell B.** Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging* 2001; **18**:685-716.
6. **Mishra DP, Dhali A.** Endotoxin induces luteal cell apoptosis through the mitochondrial pathway. *Prostaglandins Other Lipid Mediat* 2007; **83**:75-88.
7. **Nagy IZ.** On the true role of oxygen free radicals in the living state, aging, and degenerative disorders. *Ann N Y Acad Sci* 2001; **928**:187-199.
8. **Wu J, Jing L, Yuan H, Peng SQ.** T-2 toxin induces apoptosis in ovarian granulosa cells of rats through reactive oxygen species-mediated mitochondrial pathway. *Toxicol Lett* 2011; **202**:168-177.
9. **Agarwal A, Gupta S, Sharma RK.** Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005; **3**:28.
10. **Al-Gubory KH, Bolifraud P, Germain G, Nicole A, Ceballos-Picot I.** Antioxidant enzymatic defence systems in sheep corpus luteum throughout pregnancy. *Reproduction* 2004; **128**:767-774.
11. **Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM.** Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. *Free Radic Biol Med* 2008; **44**:1295-1304.
12. **Paradies G, Petrosillo G, Paradies V, Reiter RJ, Ruggiero FM.** Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease. *J Pineal Res* 2010; **48**:297-310.
13. **Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ.** One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J Pineal Res* 2007; **42**:28-42.
14. **Galano A, Tan DX, Reiter RJ.** On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. *J Pineal Res* 2013; **54**:245-257.

15. **Nakamura Y, Tamura H, Takayama H, Kato H.** Increased endogenous level of melatonin in preovulatory human follicles does not directly influence progesterone production. *Fertil Steril* 2003; **80**:1012-1016.

16. **Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan DX, Sugino N, Reiter RJ.** Melatonin and the ovary: physiological and pathophysiological implications. *Fertil Steril* 2009; **92**:328-343.

17. **Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Sugino N.** Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res* 2008; **44**:280-287.

18. **Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N.** The role of melatonin as an antioxidant in the follicle. *J Ovarian Res* 2012; **5**:5.

19. **Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Asada H, Yamagata Y, Sugino N.** Melatonin as a free radical scavenger in the ovarian follicle. *Endocr J* 2013; **60**:1-13.

20. **Reiter RJ, Paredes SD, Manchester LC, Tan DX.** Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. *Crit Rev Biochem Mol Biol* 2009; **44**:175-200.

21. **Leon J, Acuna-Castroviejo D, Escames G, Tan DX, Reiter RJ.** Melatonin mitigates mitochondrial malfunction. *J Pineal Res* 2005; **38**:1-9.

22. **Martin M, Macias M, Escames G, Leon J, Acuna-Castroviejo D.** Melatonin but not vitamins C and E maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. *FASEB J* 2000; **14**:1677-1679.

23. **Venegas C, Garcia JA, Escames G, Ortiz F, Lopez A, Doerrier C, Garcia-Corzo L, Lopez LC, Reiter RJ, Acuna-Castroviejo D.** Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. *J Pineal Res* 2012; **52**:217-227.

24. **Yamamoto HA, Mohanan PV.** Melatonin attenuates brain mitochondria DNA damage induced by potassium cyanide in vivo and in vitro. *Toxicology* 2002; **179**:29-36.

25. **Erenberk U, Dundaroz R, Gok O, Uysal O, Agus S, Yuksel A, Yilmaz B, Kilic U.** Melatonin attenuates phenytoin sodium-induced DNA damage. *Drug Chem Toxicol* 2014; **37**:233-239.

26. **Akbulut KG, Gonul B, Akbulut H.** The role of melatonin on gastric mucosal cell proliferation and telomerase activity in ageing. *J Pineal Res* 2009; **47**:308-312.

27. **Carretero M, Escames G, Lopez LC, Venegas C, Dayoub JC, Garcia L, Acuna-Castroviejo D.** Long-term melatonin administration protects brain mitochondria from aging. *J Pineal Res* 2009; **47**:192-200.

28. **Jou MJ, Peng TI, Yu PZ, Jou SB, Reiter RJ, Chen JY, Wu HY, Chen CC, Hsu LF.** Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial

oxidative stress and apoptosis. *J Pineal Res* 2007; **43**:389-403.

29. **Milczarek R, Hallmann A, Sokolowska E, Kaletha K, Klimek J.** Melatonin enhances antioxidant action of alpha-tocopherol and ascorbate against NADPH- and iron-dependent lipid peroxidation in human placental mitochondria. *J Pineal Res* 2010; **49**:149-155.

30. **Taketani T, Tamura H, Takasaki A, Lee L, Kizuka F, Tamura I, Taniguchi K, Maekawa R, Asada H, Shimamura K, Reiter RJ, Sugino N.** Protective role of melatonin in progesterone production by human luteal cells. *J Pineal Res* 2011, 51:207-213.

31. **Bekker-Jensen S, Mailand N.** Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair (Amst)* 2010; **9**:1219-1228.

32. **Ciccia A, Elledge SJ.** The DNA damage response: making it safe to play with knives. *Mol Cell* 2010; **40**:179-204.

33. **Mah LJ, El-Osta A, Karagiannis TC.** GammaH2AX as a molecular marker of aging and disease. *Epigenetics* 2010; **5**:129-136.

34. **Wu HJ, Liu C, Duan WX, Xu SC, He MD, Chen CH, Wang Y, Zhou Z, Yu ZP, Zhang L, Chen Y.** Melatonin ameliorates bisphenol A-induced DNA damage in the germ cells of adult male rats. *Mutat Res* 2013; **752**:57-67.

35. **Sun FY, Lin X, Mao LZ, Ge WH, Zhang LM, Huang YL, Gu J.** Neuroprotection by melatonin against ischemic neuronal injury associated with modulation of DNA damage and repair in the rat following a transient cerebral ischemia. *J Pineal Res* 2002; **33**:48-56.

36. **Arakane F, Kallen CB, Watari H, Foster JA, Sepuri NB, Pain D, Stayrook SE, Lewis M, Gerton GL, Strauss JF, 3rd.** The mechanism of action of steroidogenic acute regulatory protein (StAR). StAR acts on the outside of mitochondria to stimulate steroidogenesis. *J Biol Chem* 1998; **273**:16339-16345.

37. **Clark BJ, Wells J, King SR, Stocco DM.** The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J Biol Chem* 1994; **269**:28314-28322.

38. **Behrman HR, Aten RF.** Evidence that hydrogen peroxide blocks hormone-sensitive cholesterol transport into mitochondria of rat luteal cells. *Endocrinology* 1991; **128**:2958-2966.

39. **Hardeland R.** Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. *Endocrine* 2005; **27**:119-130.

40. **Reiter RJ, Paredes SD, Korkmaz A, Jou MJ, Tan DX.** Melatonin combats molecular terrorism at the mitochondrial level. *Interdiscip Toxicol* 2008; **1**:137-149.

41. **Lopez A, Garcia JA, Escames G, Venegas C, Ortiz F, Lopez LC, Acuna-Castroviejo D.** Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. *J Pineal Res* 2009; **46**:188-198.

42. **Kodaman PH, Aten RF, Behrman HR.** Lipid hydroperoxides evoke antigonadotropic and

antisteroidogenic activity in rat luteal cells. *Endocrinology* 1994; **135**:2723-2730.

43. **Sawada M, Carlson JC.** Rapid plasma membrane changes in superoxide radical formation, fluidity, and phospholipase A2 activity in the corpus luteum of the rat during induction of luteolysis. *Endocrinology* 1991; **128**:2992-2998.

44. **Wu X, Yao K, Carlson JC.** Plasma membrane changes in the rat corpus luteum induced by oxygen radical generation. *Endocrinology* 1993; **133**:491-495.

45. **Antonsson B.** Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. *Cell Tissue Res* 2001; **306**:347-361.

46. **Denault JB, Salvesen GS.** Apoptotic caspase activation and activity. *Methods Mol Biol* 2008; **414**:191-220.

47. **Tang XL, Yang XY, Jung HJ, Kim SY, Jung SY, Choi DY, Park WC, Park H.** Asiatic acid induces colon cancer cell growth inhibition and apoptosis through mitochondrial death cascade. *Biol Pharm Bull* 2009; **32**:1399-1405.

48. **Akbulut KG, Akbulut H, Akgun N, Gonul B.** Melatonin decreases apoptosis in gastric mucosa during aging. *Aging Clin Exp Res* 2012; **24**:15-20.

49. **Espino J, Bejarano I, Redondo PC, Rosado JA, Barriga C, Reiter RJ, Pariente JA, Rodriguez AB.** Melatonin reduces apoptosis induced by calcium signaling in human leukocytes: Evidence for the involvement of mitochondria and Bax activation. *J Membr Biol* 2010; **233**:105-118.

50. **Wang FW, Wang Z, Zhang YM, Du ZX, Zhang XL, Liu Q, Guo YJ, Li XG, Hao AJ.** Protective effect of melatonin on bone marrow mesenchymal stem cells against hydrogen peroxide-induced apoptosis in vitro. *J Cell Biochem* 2013; **114**:2346-2355.

51. **Niles LP, Wang J, Shen L, Lobb DK, Younglai EV.** Melatonin receptor mRNA expression in human granulosa cells. *Mol Cell Endocrinol* 1999; **156**:107-110.

52. **Soares JM, Masana MI, Ersahin C, Dubocovich ML.** Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. *J Pharmacol Exp Ther* 2003; **306**:694-702.

53. **Mayo JC, Sainz RM, Antoli I, Herrera F, Martin V, Rodriguez C.** Melatonin regulation of antioxidant enzyme gene expression. *Cell Mol Life Sci* 2002;**59**:1706-1713.

54. **Rodriguez C, Mayo JC, Sainz RM, Antoli I, Herrera F, Martin V.** Regulation of antioxidant enzymes: a significant role for melatonin. *J Pineal Res*;2004:**36**:1-9.

55. **Tamura H, Takasaki A, Taketani T, Tanabe M, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N.** Melatonin and female reproduction. *J Obstet Gynaecol Res* 2014; **40**:1-11.

Figure 1

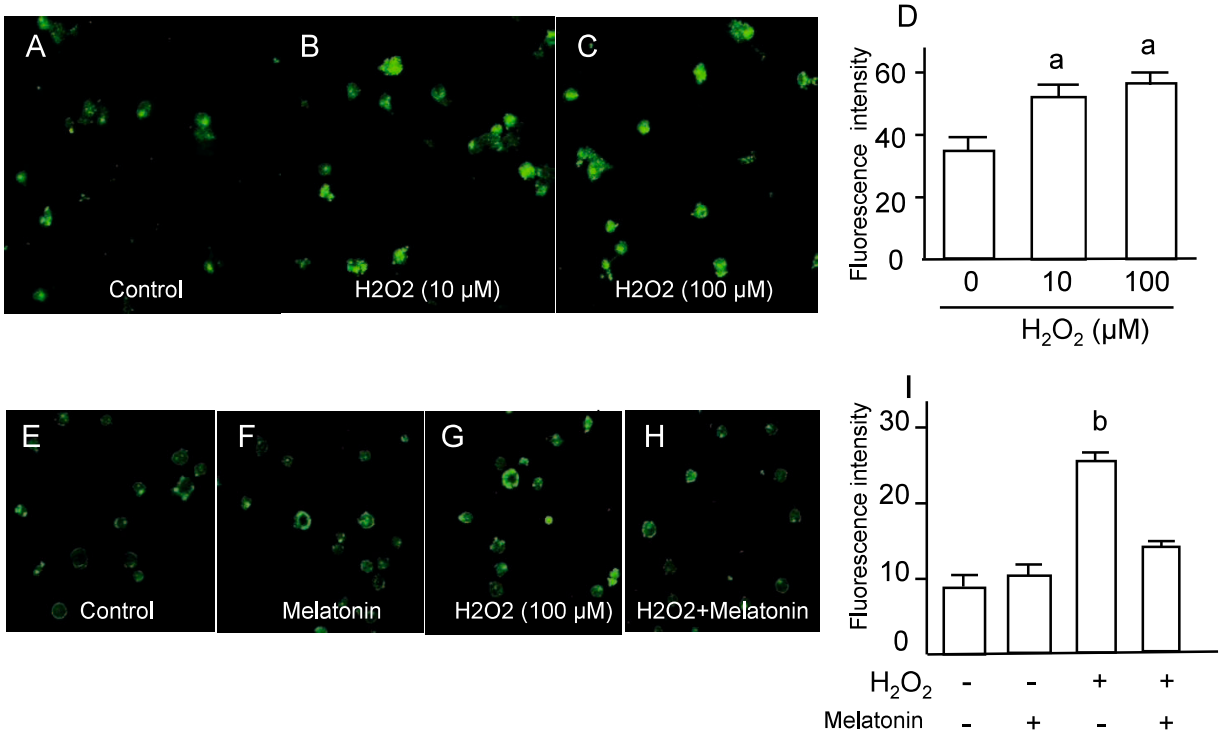

Fig. 1. Effects of H2O2 and/or melatonin on 8-Hydroxydeoxyguanosine (8-OHdG) in granulosa cells. Granulosa cells were incubated with H2O2 (0, 10, 100 μM) for 2 h. The oxidative guanine base damage of DNA was assessed by fluorescence-based immunocytochemistry using specific antibodies for 8-OHdG. (A) Control. (B) H2O2 (10 μM). (C) H2O2 (100 μM). (D) The fluorescence intensity of each group was analyzed using the CellProfiler software. Granulosa cells were also incubated with H2O2 (100 μM) in the presence or absence of melatonin (100 μg/ml) for 2 h. (E) Control. (F) Melatonin (100 μg/ml). (G) H2O2 (100 μM). (H) H2O2 (100 μM) + melatonin (100 μg/ml). (I) The fluorescence intensity of each group was analyzed as described above. Data are shown as the mean ± SEM of three independent incubations. a, $P < 0.05$ vs. control, and b, $P < 0.05$ vs. the other groups (Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction).

Figure 2

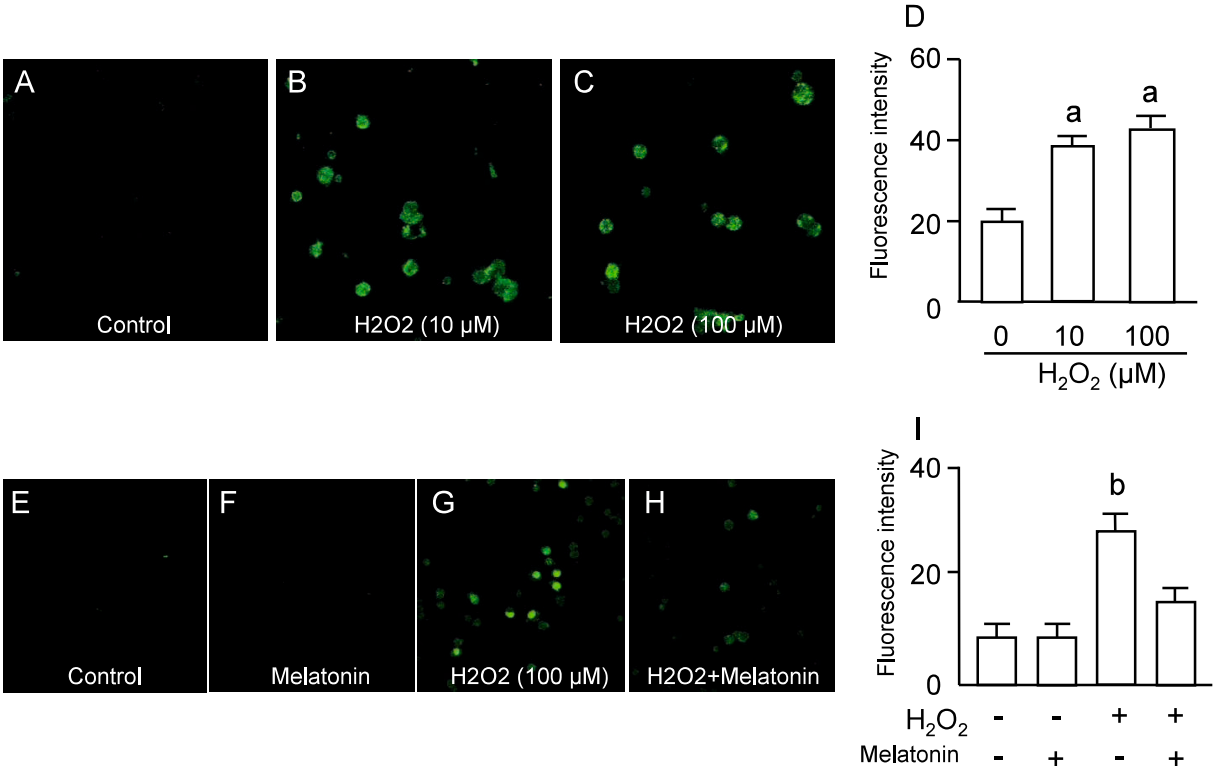

Fig. 2. Effects of H2O2 and/or melatonin on histone H2AX phosphorylation (γH2AX) in granulosa cells. Granulosa cells were incubated with H2O2 (0, 10, 100 μM) for 2 h. The double-strand breaks of DNA were assessed by fluorescence-based immunocytochemistry using specific antibodies for γH2AX. (A) Control. (B) H2O2 (10 μM). (C) H2O2 (100 μM). (D) The fluorescence intensity of each group was analyzed using the CellProfiler software. Granulosa cells were also incubated with H2O2 (100 μM) in the presence or absence of melatonin (100 μg/ml) for 2 h. (E) Control. (F) Melatonin (100 μg/ml). (G) H2O2 (100 μM). (H) H2O2 (100 μM) + melatonin (100 μg/ml). (I) The fluorescence intensity of each group was analyzed as described above. Data are shown as the mean ± SEM of three independent incubations. a, $P < 0.05$ vs. control, and b, $P < 0.05$ vs. the other groups (Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction).

Figure 3

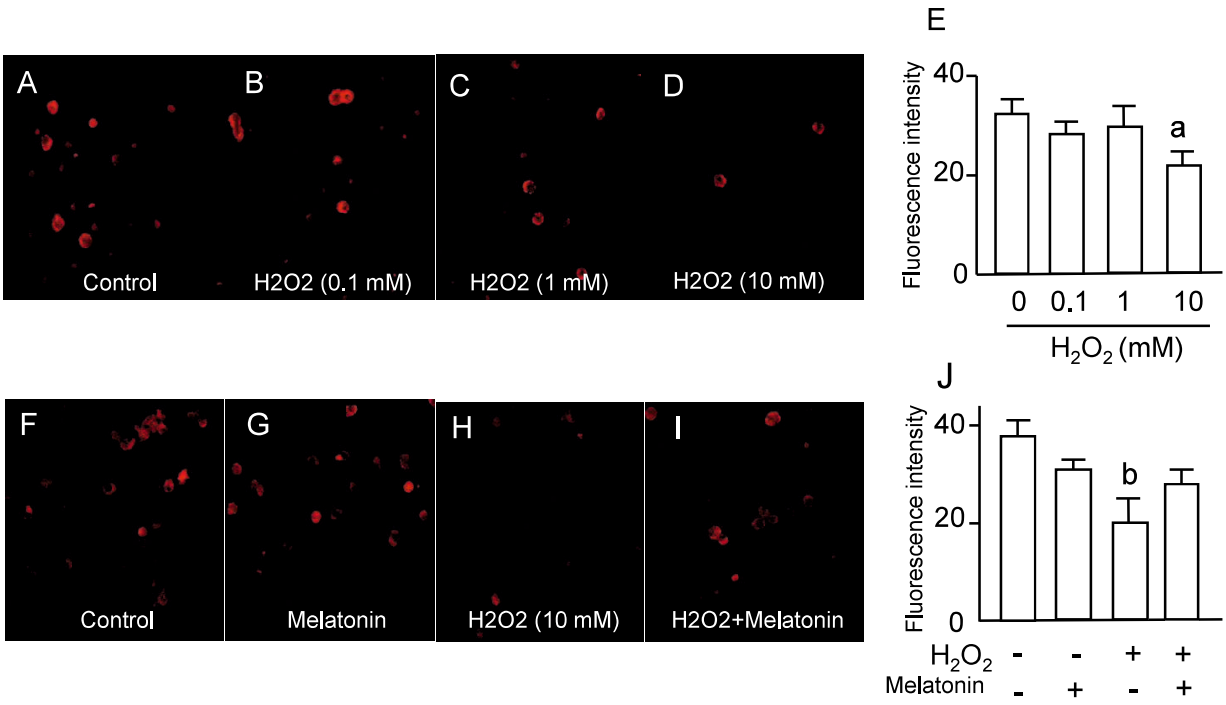

Fig. 3. Effects of H2O2 and/or melatonin on mitochondrial function. Granulosa cells were incubated with H2O2 (0.1, 1, 10 mM) for 2 h, and then the cells were loaded with a mitochondrial-targeted fluorescent probe, MitoTracker Red, at a concentration of 100 nM for 15 min. The fluorescence images were obtained using a confocal laser scanning microscope. (A) Control. (B) H2O2 (0.1 μM). (C) H2O2 (1 mM). (D) H2O2 (10 mM). (E) The fluorescence intensity of each group was analyzed using the ImageJ software. Granulosa cells were also incubated with H2O2 (10 mM) in the presence or absence of melatonin (100 μg/ml) for 2 h. (F) Control. (G) Melatonin (100 μg/ml). (H) H2O2 (10 mM). (I) H2O2 (10 mM) + melatonin (100 μg/ml). (J) The fluorescence intensity of each group was analyzed as described above. Data are shown as the mean ± SEM of three independent incubations. a, $P < 0.05$ vs. control, and b, $P < 0.05$ vs. the other groups (Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction).

Figure 4

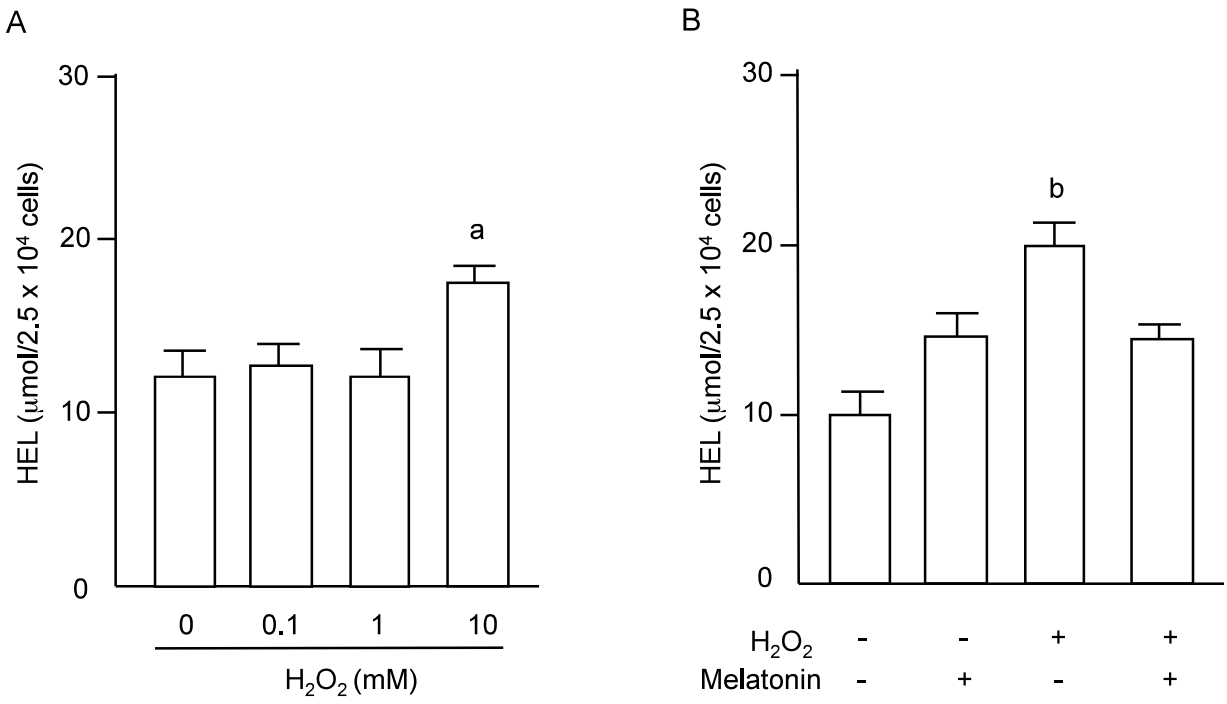

Fig. 4. Effects of H2O2 and/or melatonin on lipid peroxidation. (A) Granulosa cells were incubated with H2O2 (0.1, 1, 10 mM) for 2 h. (B) Granulosa cells were incubated with H2O2 (10 mM) in the presence or absence of melatonin (100 μg/ml) for 2 h. Lipid peroxidation of cell membranes was analyzed by measuring hexanoyl-lysine (HEL). Data are shown as the mean ± SEM of three independent incubations. a, $P < 0.05$ vs. control, and b, $P < 0.05$ vs. the other groups (Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction).

Figure 5

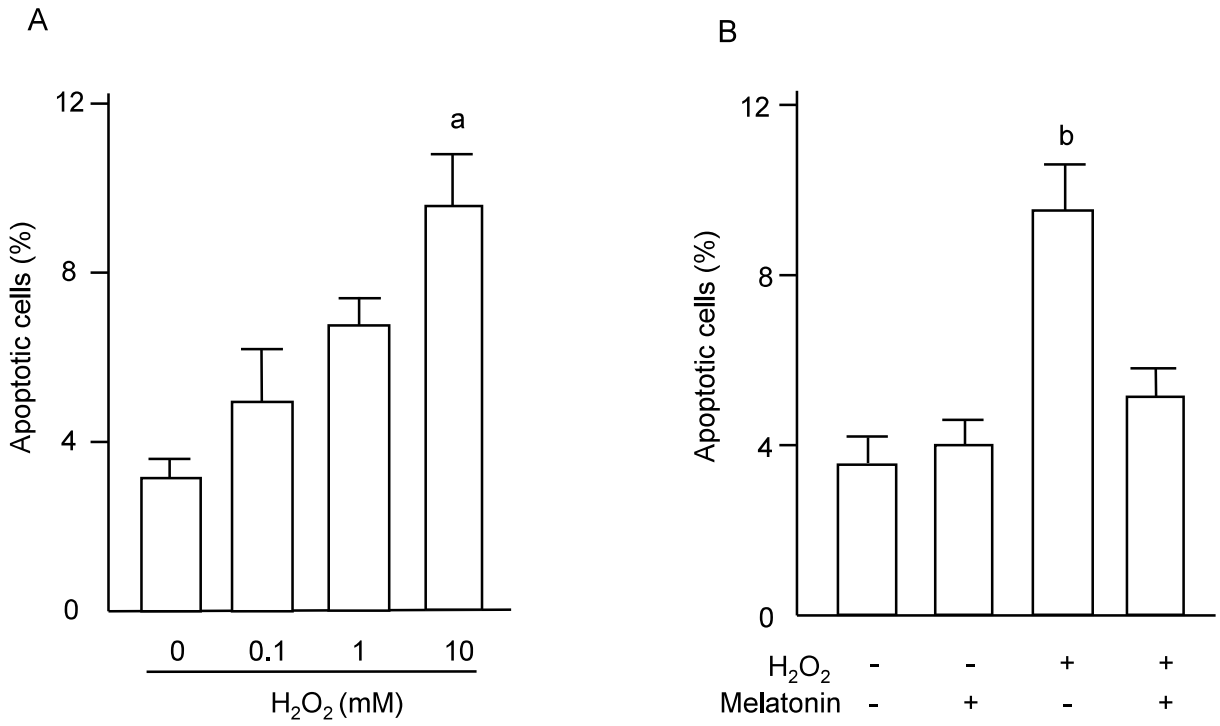

Fig. 5. Effects of H2O2 and/or melatonin on apoptosis of granulosa cells. (A) Granulosa cells were incubated with H2O2 (0.1, 1, 10 mM) for 2 h. (B) Granulosa cells were incubated with H2O2 (10 mM) in the presence or absence of melatonin (100 μg/ml) for 2 h. Apoptosis of granulosa cells was assessed by nuclear fragmentation using DAPI staining. Apoptotic cells were identified by condensation and fragmentation of the nuclei. Apoptosis was quantified by calculating the percentage of apoptotic nuclei in a total of 200 nuclei in each group. Data are shown as the mean ± SEM of three independent incubations. a, $P < 0.05$ vs. control, and b, $P < 0.05$ vs. the other groups (Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction).

Figure 6

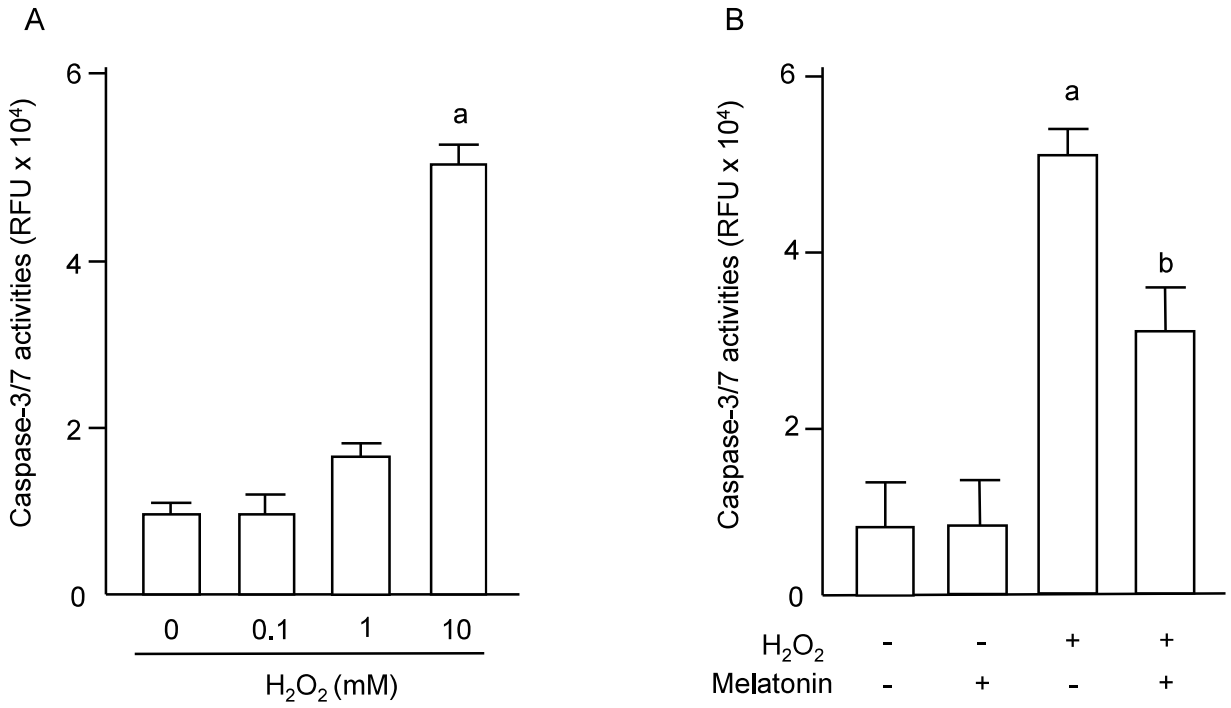

Fig. 6. Effects of H2O2 and/or melatonin on caspase-3/7 activities of granulosa cells. (A) Granulosa cells were incubated with H2O2 (0.1, 1, 10 mM) for 2 h. (B) Granulosa cells were incubated with H2O2 (10 mM) in the presence or absence of melatonin (100 μg/ml) for 2 h. Caspase-3/7 activities were determined as overall activities in 2.5×10^4 granulosa cells and shown as relative fluorescence units (RFU). Data are shown as the mean ± SEM of three independent incubations. a, $P < 0.01$ vs. control, and b, $P < 0.05$ vs. H2O2 (Kruskal-Wallis H-test and Mann-Whitney U-test with Bonferroni correction).