

## Protective Effect of Melatonin on LPS-stimulated Granulosa Cells in Japanese Quail

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The aim of this study was to evaluate the potential of melatonin to protect cultured granulosa cells from the harmful effects of lipopolysaccharide (LPS) in quail. Granulosa cells isolated from Japanese quails were pretreated with or without melatonin (10 or 100  $\mu\text{g}/\text{mL}$ ) for 12 h and then incubated for 12 h in the absence or presence of 100 ng/mL LPS. The expression of pro-inflammatory cytokines and chemokine was detected by quantitative real-time PCR. The levels of oxidative stress biomarkers (dityrosine and nitrite) were determined by ELISA and the Griess reaction. Cell viability was quantified using an MTT assay. Additionally, the level of progesterone was measured by ELISA. We found that melatonin decreased LPS-induced expression of *IL-1 $\beta$* , *IL-6*, and *IL-8*. In addition, melatonin increased the dityrosine level, but suppressed the nitrite level. Finally, melatonin administration increased the viability of LPS-stimulated granulosa cells *in vitro*. However, progesterone basal secretion was not significantly changed. These results suggest that melatonin protects cultured granulosa cells from LPS-induced inflammatory and oxidative stress damage and provide evidence that melatonin might have therapeutic utility in ovarian follicle infection in Japanese quail.

**Key words:** cytokine, granulosa cell, lipopolysaccharide, melatonin, oxidant stress

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### Introduction

Infections of the reproductive tract not only cause substantial economic losses in the poultry industry, but also threaten public health (Wigley *et al.*, 2005). Ovarian follicles infected with gram-negative bacteria cause ovarian disorders and the production of contaminated eggs (Abdelsalam *et al.*, 2011). *Escherichia coli* is one of the main pathogens causing this problem. The innermost follicular wall surrounding the yolk is the granulosa layer, and bacteria can invade this layer (Takata *et al.*, 2003). Accumulation of the bacterial endotoxin lipopolysaccharide (LPS) in the egg yolk of animals is associated with ovarian dysfunction (Wigley *et al.*, 2005). The local immune system plays an essential role in host defense. Pro-inflammatory cytokines initiate innate and adaptive immune responses and assist in generating a local inflammatory response (Hughes *et al.*, 2007).

The extensive use of antibiotics in animal production raises consumer concern over food safety (Yu *et al.*, 2016).

At the same time, with the growing world population, more and better-quality food will be needed (Yu and Maeda, 2017). Melatonin (*N*-acetyl-5-methoxytryptamine), a circadian hormone (Pang *et al.*, 1998), has been recently reported to be involved in various physiological processes, such as female reproduction (Wang *et al.*, 2014; Yu *et al.*, 2017), innate immunity (Zhou *et al.*, 2016), abiotic stress resistance (Zhang *et al.*, 2015), and antiradiation (Fernández-Gil *et al.*, 2017), anticancer (Moreira *et al.*, 2015; Söderquist *et al.*, 2016), and antioxidant processes (Mehaisen *et al.*, 2015) in both plants and animals. Besides melatonin, its metabolites, including cyclic-3-hydroxymelatonin, *N*-acetyl-*N*-formyl-5-methoxykynuramine, and *N*-acetyl-5-methoxykynuramine, also have the ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Galano *et al.*, 2014). As the inflammatory response is typically accompanied by free radical generation, the reduction in free radical-mediated damage by melatonin contributes to its anti-inflammatory effects (Mauriz *et al.*, 2013). A number of studies have demonstrated the anti-inflammatory activity of melatonin both *in vivo* and *in vitro*. Melatonin reduces oxidative damage and suppresses *IL-6* mRNA expression after venous infusion of LPS and peptidoglycan in rats (Lowe *et al.*, 2013), and it diminishes the level of *IL-1 $\beta$*  in different

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regions of the rat brain after intracerebroventricular administration of LPS (Tyagi *et al.*, 2010). Melatonin treatment also inhibited the expression of IL-8 and oxidative stress mediators in LPS-stimulated RAW264.7 cells *in vitro* (Xia *et al.*, 2012).

Previous studies have shown the ability of melatonin to repress pro-inflammatory cytokine levels and reduce oxidative stress in experimental inflammation. However, to our knowledge, the anti-inflammatory effect of melatonin on granulosa cells has not been investigated. Here, we conducted a series of experiments to determine whether melatonin can protect cultured granulosa cells from LPS-induced cell damage in Japanese quail. We investigated the effects of melatonin on the mRNA levels of pro-inflammatory cytokines and chemokine in LPS-stimulated granulosa cells *in vitro*. Additionally, we examined ROS/RNS levels in the cell culture medium and evaluated the potential benefits of melatonin on cell viability following LPS treatment. Finally, we investigated the effect of high doses of melatonin on progesterone basal secretion by granulosa cells.

## Materials and Methods

### Experimental Birds

In total, nine female Japanese quails, 15–30 weeks of age, were used. Six quails were used for analyses by PCR, ELISA, and the Griess reaction; the other three quails were used for MTT assay. All quails were reared in individual cages under a lighting regimen of 14 h light:10 h dark and were provided with feed and water *ad libitum*. Birds were decapitated to collect the largest pre-ovulatory (F1) follicles at 18–21 h after oviposition. All animals used in this study were handled in accordance with the regulations of Animal Experiment Committee of Hiroshima University for animal experiments.

### Cell Culture and Treatment

Granulosa cells were isolated in aseptic conditions as previously described (Rangel *et al.*, 2009). Briefly, the largest pre-ovulatory (F1) follicle was placed in cell culture dishes containing Dulbecco's phosphate-buffered saline (DPBS; Nissui Pharmaceutical, Tokyo, Japan). After the yolk was drained out through an incision made in the follicular wall, the follicle was inverted and shaken in DPBS. The granulosa layer detached from the theca was disaggregated at 37°C for 5 min under continuous agitation in 500  $\mu$ L of dissociation solution (CTK; ReproCELL, Yokohama, Japan), and then flushed with a pipette. The enzymatic solution was quenched by the addition of 2 mL of DMEM/F12 medium containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). The cell suspensions were centrifuged, and the pelleted cells were washed three times by resuspension in 5 mL DPBS and centrifugation for 5 min at 500  $\times$  g. The viability of the granulosa cells was approximately 97% as determined by trypan blue dye exclusion. The cells were seeded in 6-well culture plates (NUNC, Roskilde, Denmark) at  $1 \times 10^6$  viable cells per well in 2.5 mL DMEM/F12 medium (Gibco BRL/Invitrogen, Karlsruhe, Germany) containing 10% FBS, 1% nucleosides (Millipore,

Billerica, MA, USA), 1% non-essential amino acids (Gibco BRL/Invitrogen), 1 mM sodium pyruvate (Gibco BRL/Invitrogen), and 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan). The cells were cultured in a humidified atmosphere with 5% CO<sub>2</sub> and 95% air at 39°C.

A stock solution of melatonin (Sigma-Aldrich) at 50 mg/mL in ethanol was prepared. Working solutions were prepared by dilution in cell culture medium to yield final concentrations of 10 and 100  $\mu$ g/mL, according to a previous study (Xia *et al.*, 2012). Granulosa cells were pretreated with or without melatonin for 12 h and then incubated with 100 ng/mL LPS (from *E. coli*; ALX-581-014-L001; Enzo Life Sciences, Farmingdale, NY, USA) (Shao *et al.*, 2015) for another 12 h, a culture time used previously (Taketani *et al.*, 2011). As controls, cells were incubated with ethanol as the solvent, at the highest concentration used in the melatonin treatments.

### RNA Isolation and cDNA Preparation

Total RNA was extracted using NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. The RNA was quantified by measuring the optical density at a wavelength of 260 nm using an OD260 unit equivalent to 40  $\mu$ g/mL of RNA. The RNA purity was determined by measuring the absorbance ratio at 260/280 nm. Only samples with a ratio between 1.8 and 2.2 were used. Aliquots of 240 ng of total RNA were reverse-transcribed to cDNA using the ReverTra Ace kit (Toyobo, Osaka, Japan).

### Quantitative PCR

Quantitative PCR was performed using the SYBR Premix Ex Taq II kit (Takara Bio, Shiga, Japan) on an Applied Biosystems StepOne Real-Time PCR System (Life Technologies, Darmstadt, Germany) according to the method described previously (Guangmin *et al.*, 2015). Briefly, the PCR mixture consisted of 10  $\mu$ L SYBR Premix Ex Taq II, 0.4  $\mu$ M forward and reverse primers, 0.4  $\mu$ L ROX reference dye, and 2  $\mu$ L template, and ddH<sub>2</sub>O was added to a total volume of 20  $\mu$ L. The thermal cycles were as follows: initial denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 34 s, and melting from 60 to 95°C, increasing in increments of 0.5°C every 5 s. Normalization was done using the housekeeping gene *GAPDH*. Primer sequences are listed in Table 1. Relative mRNA expression was calculated by the  $2^{-\Delta\Delta C_t}$  method. Samples from six quails were analyzed.

### ELISA

Cell culture medium was precleared by centrifugation at 3,000  $\times$  g for 20 min to remove cells and stored at  $-20^\circ\text{C}$  until use. Dityrosine concentration in samples of culture medium was measured using a competitive ELISA kit (JaICA; Nikken SEIL, Shizuoka, Japan), according to the manufacturer's protocol. Dityrosine levels were calculated with reference to a standard curve that typically ranged from 0.05 to 12  $\mu$ M. Samples from six quails were analyzed in duplicate.

For the progesterone assay, cell-free culture medium was

Table 1. Primers used for qRT-PCR

Gene	Primer sequence (5'-3')	Accession no.	Product size (bp)
<i>IL-1<math>\beta</math></i>	Forward: GGGCATCAAGGGCTACAA	NM_204524.1	138
	Reverse: CTGTCCAGGCGGTAGAAGAT		
<i>IL-6</i>	Forward: AGAAATCCCTCCTCGCCAAT	NM_204628.1	121
	Reverse: AAATAGCGAACGGCCCTCA		
<i>IL-8</i>	Forward: GGCTTGCTAGGGGAAATGA	HM179639.1	200
	Reverse: AGCTGACTCTGACTAGGAAACTGT		
<i>GADPH</i>	Forward: ATCACAGCCACACAGAAGACG	M11213	124
	Reverse: TGACTTTCCCCACAGCCTTA		

prepared as described previously (Isobe *et al.*, 2007). An anti-progesterone antibody was produced in rabbits (Isobe and Nakao, 2003). Horseradish peroxidase was conjugated with progesterone carboxymethyloxime (Sigma-Aldrich) using a mixed anhydride reaction. Cross reactivities of the anti-progesterone antibody with progesterone, 5 $\alpha$ -pregnenedione, 20 $\beta$ -hydroxyprogesterone, deoxycorticosterone, pregnenolone, 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one, and 17 $\alpha$ -hydroxyprogesterone were 100, 5.8, 0.7, 0.62, 0.2, 0.1, and 0.05%, respectively. The sensitivity of the assay was 0.0055 ng/mL. Intra- and inter-assay coefficients of variation were 9.6–10.9 % and 10.8–16.6%, respectively. Recovery rate ranged between 73 and 84%. Samples from six quails were analyzed in duplicate.

#### Griess Reaction

The level of the nitric oxide (NO) metabolite nitrite in samples of cell culture medium was measured with a nitrite colorimetric assay kit (Dojindo Molecular Technologies, Tokyo, Japan) based on the Griess reaction, according to the manufacturer's instruction. Samples were measured using a spectrophotometer (Model 680 microplate reader S/N 22002; Bio-Rad Laboratories, Hercules, CA, USA), and nitrite concentrations were calculated by comparison with a standard curve. Samples from six quails were analyzed in duplicate.

#### MTT Assay

The viability of cultured granulosa cells was quantified using an MTT assay. Briefly, cells were seeded in 96-well plates at  $1 \times 10^5$  cells/well and pretreated with or without melatonin for 12 h and then stimulated for 12 h with 100 ng/mL LPS. The cells were then washed three times with DPBS, and fresh culture medium containing 5 mg/mL MTT (Sigma-Aldrich) was added to each well. After incubation at 37°C for 4 h, the culture medium was removed and 100  $\mu$ L dimethyl sulfoxide (Nacalai Tesque) was added. The plates were oscillated for 15 min, and the absorbance at 570 nm was measured spectrophotometrically (Model 680 microplate reader S/N 22002; Bio-Rad Laboratories). Samples from three quails were analyzed in sextuplicate.

#### Statistical Analysis

Continuous variables are expressed as the mean  $\pm$  SD of 3–6 independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test with StatView software (Abacus Concepts, Berkeley, CA, USA). A *P*-value

less than 0.05 was considered statistically significant.

## Results

LPS caused substantial increases in the mRNA levels of *IL-1 $\beta$* , *IL-6*, and *IL-8* (Fig. 1a–c). Addition of 10  $\mu$ g/mL melatonin significantly decreased *IL-1 $\beta$*  mRNA level ( $P < 0.05$ ) (Fig. 1a), whereas *IL-6* and *IL-8* mRNA levels were significantly decreased by melatonin at concentrations of 10 and 100  $\mu$ g/mL ( $P < 0.05$ ) (Fig. 1b and c).

To examine oxidative stress, we measured the dityrosine level in the supernatants of treated granulosa cells. ELISA showed that LPS substantially decreased the production of dityrosine ( $P < 0.05$ ), but this effect was inhibited by pretreatment with 10 and 100  $\mu$ g/mL melatonin (Fig. 2).

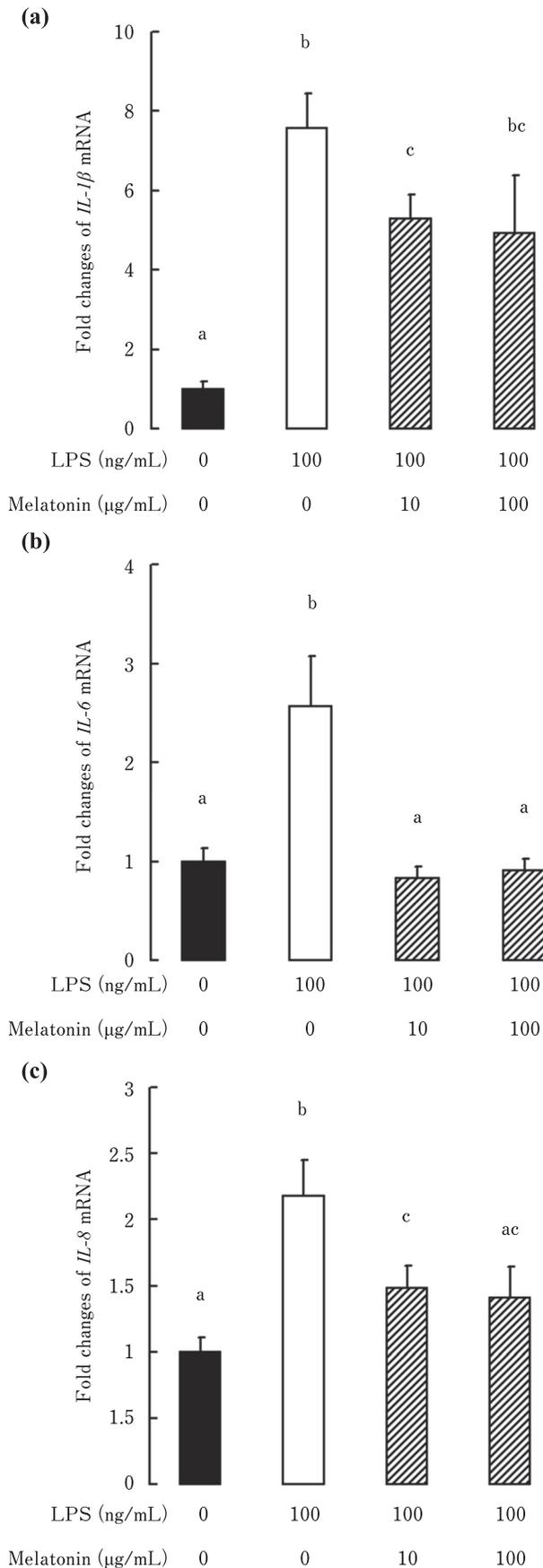
To evaluate nitrate stress, the level of nitrite in the culture medium was determined by the Griess reaction; it was markedly increased by LPS stimulation ( $P < 0.05$ ) (Fig. 3). As expected, melatonin significantly suppressed the nitrite levels at both 10 and 100  $\mu$ g/mL ( $P < 0.05$ ).

We examined the viability of cultured granulosa cells using the MTT assay. We found that although LPS significantly reduced the cell viability ( $P < 0.05$ ), melatonin at concentrations of 10 and 100  $\mu$ g/mL prevented cell death (Fig. 4).

The basal secretion of progesterone was checked by ELISA. No significant differences were detected between any of the treatments ( $P > 0.05$ ) (Fig. 5).

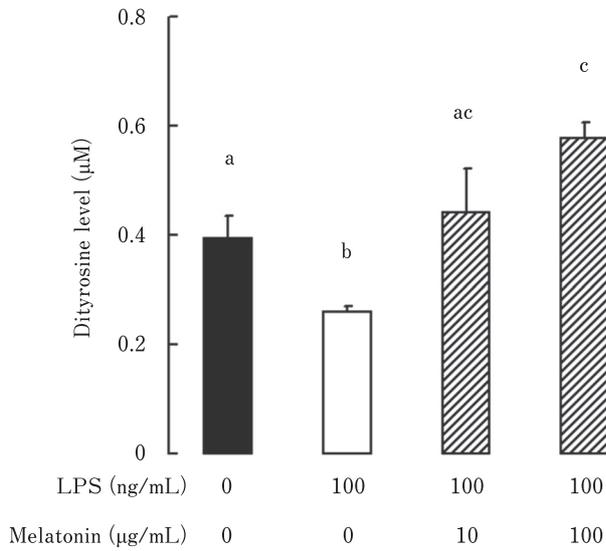
## Discussion

Cytokines are a broad and loose category of small, non-structural, secreted proteins that are synthesized by and induce a response in nearly all nucleated cells (Dinarello, 2000). They are primarily involved in host responses to infection, trauma, and cancer, and also play roles in reproduction. Considerable attention has focused on blocking the action of pro-inflammatory cytokines, particularly during overwhelming infection. Previous studies have revealed that melatonin diminishes *IL-1 $\beta$* , *IL-6*, and *IL-8* during bacterial infections or LPS treatment *in vitro* and *in vivo* (Tyagi *et al.*, 2010; Xia *et al.*, 2012; Lowes *et al.*, 2013). The results of the present study corroborate that melatonin decreases the mRNA expression of *IL-1 $\beta$* , *IL-6*, and *IL-8* in LPS-stimulated granulosa cells, which is likely to have a beneficial effect by reducing inflammatory responses.

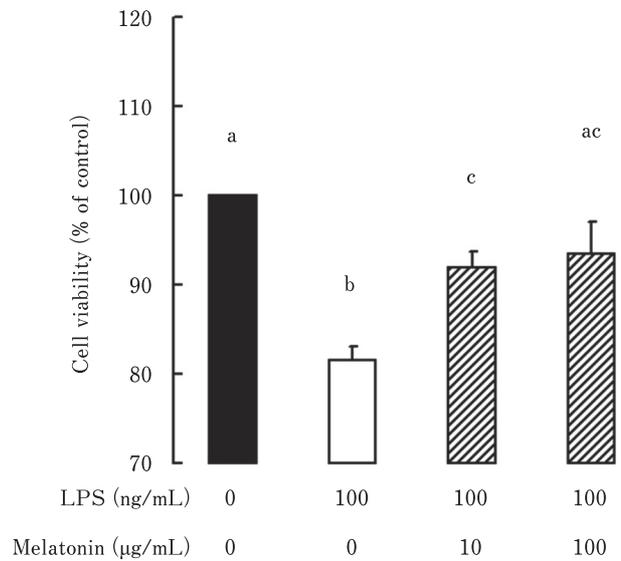


An inflammatory response activates the synthesis of immunomodulators, which results in cataclysmic production of ROS and RNS (Reuter *et al.*, 2010). These toxicants trigger the discharge of sequestered  $\text{Ca}^{2+}$  into the cytosol and cause mitochondrial lesions, which results in the release of cytochrome c and activation of the apoptotic cascade (Reiter *et al.*, 2016). An effective anti-inflammatory therapeutic strategy thus requires reducing the production of inflammatory mediators, as well as abating the oxidative stress initiated by inflammation (Radogna *et al.*, 2010). As a powerful antioxidant, melatonin represses oxidative stress by direct scavenging of free radicals, stimulation of antioxidant enzymes, and chelation of transition metals (Reiter *et al.*, 2016). As a result, melatonin assuages oxidative stress-related pathologies, reduces cellular apoptosis, and preserves cell function. In this study, melatonin successfully reversed the LPS-induced reduction in dityrosine levels and suppressed nitrite levels. Dityrosine is generated by tyrosine dimerization during normal post-translational processes. Because the dimerization and nitration of tyrosine can be affected by peroxynitrite, dityrosine is considered a biomarker of oxidatively modified proteins (Hattori *et al.*, 2015). It has been reported that tyrosine is almost exclusively dimerized to give dityrosine at peroxynitrite levels of  $5\mu\text{M}$  or less, whereas the reaction progressively shifts toward nitration at higher peroxynitrite concentrations (Pfeiffer *et al.*, 2000). Nitration of tyrosine residues on proteins is associated with peroxynitrite-mediated tissue injury under severe inflammatory conditions (Souler *et al.*, 2001). Thus, the inhibition of the LPS-induced decrease in dityrosine levels by melatonin pretreatment in our study suggests that melatonin reduces oxidative protein damage. NO is produced from L-arginine by the enzyme NO synthase. It is an important intracellular and extracellular signaling molecule that is involved in diverse biological processes, including regulation of vascular tone, neurotransmission, immune response, and inhibition of platelet aggregation (Andruxhov *et al.*, 2013). NO is also an important cytotoxic mediator under pathological conditions. It reacts with oxygen, superoxide anions, and reducing agents to generate products with nitrosative toxicity (Hughes, 2008). A decrease in the NO level is beneficial for cells, precluding LPS action and reducing inflammation. NO released by cultured cells into the medium is easily oxidized to nitrite (Pinho *et al.*, 2011). It is well known that nitrite levels are markedly increased under inflammatory conditions (Pinho *et al.*, 2011). Our results show that level of nitrite in the culture medium of LPS-stimulated granulosa cells was

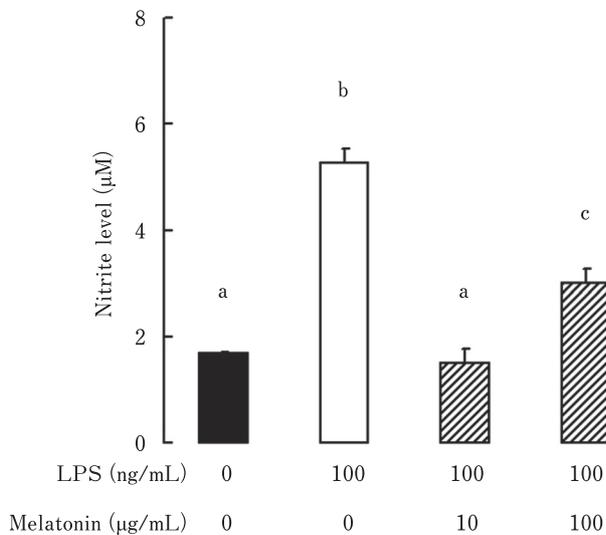
**Fig. 1. Effect of melatonin on LPS-induced cytokine and chemokine mRNA expression.** Melatonin modulates LPS-induced cytokine mRNA expression. qRT-PCR analysis of (A) *IL-1β*, (B) *IL-6*, and (C) *IL-8* mRNA in cultured granulosa cells treated with the indicated combinations of LPS and melatonin. Data are the mean  $\pm$  SD of six independent experiments. Values with different superscripts are significantly different at  $P < 0.05$ .



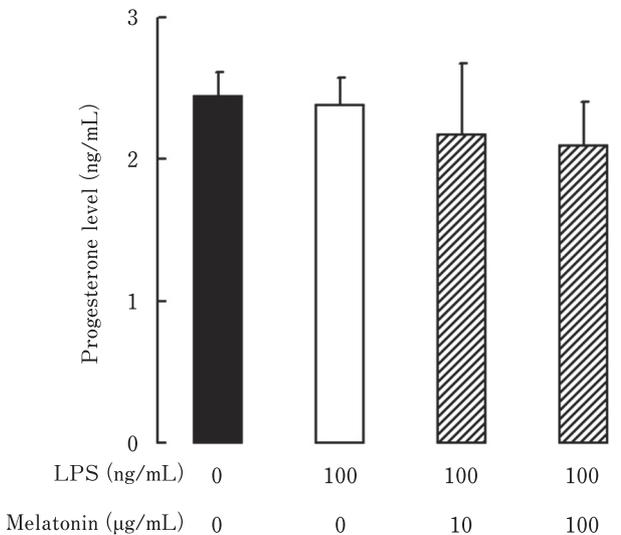
**Fig. 2. Effect of melatonin on dityrosine level in LPS-stimulated granulosa cells.** Cells were treated with the indicated combinations of LPS and melatonin, and culture supernatants were analyzed for the presence of dityrosine by ELISA. Data are the mean  $\pm$  SD of six independent experiments, each measured in duplicate. Values with different superscripts are significantly different at  $P < 0.05$ .



**Fig. 4. Effect of melatonin on LPS-induced cell death.** Cell viability was quantified using the MTT assay. Data are the mean  $\pm$  SD of three independent experiments, each measured in sextuplicate. Values with different superscripts are significantly different at  $P < 0.05$ .



**Fig. 3. Effect of melatonin on nitrite level in LPS-stimulated granulosa cells.** Cells were treated with the indicated combinations of LPS and melatonin, and culture supernatants were analyzed for the presence of nitrites by the Griess reaction. Data are the mean  $\pm$  SD of six independent experiments, each measured in duplicate. Values with different superscripts are significantly different at  $P < 0.05$ .



**Fig. 5. Progesterone secretion by cultured granulosa cells.** The level of progesterone was measured by ELISA. Data are the mean  $\pm$  SD of six independent experiments, each measured in duplicate.

significantly suppressed by pretreatment with melatonin. Therefore, melatonin prevented nitrosative stress caused by LPS stimulation *in vitro*. Our findings are consistent with a previous observation that modulation of apoptosis requires high melatonin doses (Radogna *et al.*, 2009). Xia *et al.* (2012) showed that melatonin at 10–232  $\mu\text{g}/\text{mL}$  effectively inhibited LPS-induced inflammation. Both concentrations of 10 and 100  $\mu\text{g}/\text{mL}$  melatonin used in the present study inhibited cell death, which is likely to promote rehabilitation. Progesterone plays a key role in regulating reproductive activity in birds (Ito *et al.*, 2011); thus, it is important to evaluate the effect of high melatonin doses on progesterone secretion. Our results indicated that the basal secretion of progesterone by cultured granulosa cells was not influenced by either LPS or melatonin in the indicated combinations. However, luteinizing hormone-stimulated progesterone production by granulosa cells under inflammatory conditions needs further investigation.

In summary, we found that melatonin treatment of cultured granulosa cells attenuated LPS-stimulated increases in the levels of pro-inflammatory cytokines and chemokine, and oxidative stress in Japanese quail. In addition, melatonin treatment had a beneficial effect on the viability of LPS-stimulated granulosa cells *in vitro*.

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