Doctoral Thesis

Studies on the Utilization of Melatonin for Livestock Production

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Contents

Chapter 1

General introduction

Good health is essential for good performance and the welfare of livestock (Sutherland *et al.*, 2013). Health can be influenced by the interaction between the innate adaptive components of the immune system and other factors, such as the local and systemic inflammatory response, which can sometimes be more harmful than useful (Bertoni *et al.*, 2015). Such inflammation impairs livestock performance by lowering milk yield, dry matter intake, energy efficiency and fertility (Sundrum, 2015). Thus, for livestock, it is important to avoid, or reduce as much as possible, any kind of infectious and the associated inflammation. Antibiotics have been used since more than 70 years ago and have led to an attractive reduction in illness and death from infectious diseases (Fair and Tor, 2014). Contemporarily, approximately 80% of the antibiotics sold in the United States are used in meat and poultry production (Slaughter, 2011); about 70% of these are medically important, i.e., from classes important to human medicine (Food and Drug Administration, 2014). Since antibiotics have been used so widely and for so long, antibiotic resistance has become a major public health threat and it is growing (Centers for Disease Control and Prevention, 2012). Humans are at risk both due to potential presence of superbugs in animal products, and to the general migration of superbugs into the environment,

where they can transmit their genetic immunity to antibiotics to other bacteria, including pathogenic bacteria (Consumers Union, 2012).

The practice of medicine and public health would be catastrophically affected if antibiotics were not generally effective in treating bacterial diseases (Martin *et al.*, 2015). To the degree that antibiotic overuse in food animals aggravates problems with resistance; this overuse is a factor causing to the increased costs to treat antibiotic-resistant infections in humans (O'Neill, 2015). According to the Infectious Diseases Society of America (2011), longer and more expensive hospital stays for treating antibiotic resistance cost the US health care sector an estimated $21 to $34 billion and more than 8 million additional hospital days annually. Health organizations, including the World Health Organization, agree and have called for significant reductions in the use of antibiotics for animal food production (Aarestrup, 2015). In the future, ways to reduce inflammation while maintaining a good immune defense for livestock must be developed.

Some beneficial effects of the currently used alternatives to antibiotics, i.e. probiotics (Ellinger *et al.*, 1978; Hofacre *et al.*, 1998; La Ragione *et al.*, 2004), prebiotics (Oyofo *et al.*, 1989; Fukata *et al.*, 1999; Schepetkin and Quinn, 2006; Novak and Vetvicka, 2008), organic acids (Thompson and Hinton, 1997; Van Immerseel *et al.*, 2006), phytogenics (Waghorn *et al.*, 1987; Killeen *et al.*, 1998; Burt, 2004) and zeolites (Fratric *et al.*, 2005; Katsoulos *et al.*, 2006; Fratric *et al.*, 2007; Dedousi *et al.*, 2008; Gvozdic *et al.*, 2008; Pourliotis *et al.*, 2012) on health as well as on production performance of the livestock have been well studied. Recent years, the

anti-inflammatory effect of melatonin has been highly focused on.

Melatonin (N-acetyl-5-methoxytryptamine) was first isolated from bovine pineal gland more than half a century ago (Lerner *et al.*, 1958). It is the main chronobiotic hormone that regulates the circadian rhythms and seasonal changes in vertebrate physiology via its daily nocturnal increase in the blood (Reiter, 1991). Its biosynthesis from tryptophan involves four well-defined intracellular steps catalyzed by tryptophan hydroxylase, aromatic amino acid decarboxylase, arylalkylamineN-acetyltransferase, and hydroxyindole-O-methyltransferase (Stehle *et al.*, 2011). The remarkable functional versatility of melatonin is reflected in its wide distribution within phylogenetically distant organisms including bacteria, unicellular eukaryotic organisms, invertebrates and vertebrates, algae, plants and fungi, and is also found in various edibles, such as vegetables, fruits, herbs and seeds (Hardelan and Poeggeler, 2003; Carrillo-Vico *et al.*, 2013). Additionally, melatonin shows a remarkable functional versatility in both plants and animals, exhibiting antioxidant (Poeggeler *et al.*, 1994; Reiter *et al.*, 2016), oncostatic (Lissoni *et al.*, 1993), antiaging (Poeggeler *et al.*, 2005), chronobiotic actions (Peschke *et al.*, 2015), female reproduction (Wang *et al.*, 2012), innate immunity (Carrillo-Vico *et al.*, 2005; Zhou *et al.*, 2016), abiotic stress resistance (Zhang *et al.*, 2015), anti-cancer (Moreira *et al.*, 2015; Söderquist *et al.*, 2016), and antiradiative effects (Fernández-Gil *et al.*, 2017).

With regard to the melatonin mechanisms of action, four models have been described: (i) by binding to membrane receptors (Dubocovich, 1995), (ii) via an interaction with nuclear receptors (Wiesenberg *et al.*, 1995), (iii) via an interaction

with cytoplasmic proteins (Benitez-King *et al.*, 1993), and (iv) via direct, receptor-independent actions (Korkmaz *et al.*, 2009). The large spectrum of functions of melatonin can be divided into chronobiotic and nonchronobiotic functions. Chronobiotic effects are mediated by the daily rhythm of melatonin in the serum due to nocturnal pineal synthesis, whereas the melatonin produced by other cells, such as immunocompetent and gastrointestinal cells, is independent of the light/dark cycle and exerts nonchronobiotic effects (Cecon and Markus, 2011).

A large number of studies involving a definition of relationships between nervous, endocrine, and immune systems have shown one of the most attractive discoveries in modern biology that these systems use a common chemical language for intra- and intersystem communication (Blalock, 2005). Currently, pineal-synthesized melatonin is considered one of the members of the complex neuroendocrine-immunological network, and the existence of a bidirectional communication between the pineal gland and the immune system is accepted. The neuroimmunomodulatory effect of melatonin on the immune system is supported by the existence of specific melatonin receptors in immune organs as well as immunocompetent cells. These melatonin receptors are located both in plasma membrane and in nucleus of the cells.

It is interesting to note that, as an immune modulator, melatonin can exert both pro- and anti-inflammatory effects, which seems to largely depend on the cells and systems studied, and especially to the grade of inflammation. The pro-inflammatory effects seem to be observed under basal conditions, whereas the anti-inflammatory

effects are observed in the presence of high-grade inflammation (Hardeland *et al.*, 2015). Thus, melatonin appears to act as a buffer, allowing the immune system to respond to infections while attenuating serious damage in high-grade inflammation (Carrillo-Vico *et al.*, 2013). It is also thought that melatonin may promote the early stages of inflammation but suppress the sustained response to prevent chronic inflammatory disease (Barrett *et al.*, 2010). In fact, the application of melatonin to treat inflammation derives from previous researches where melatonin doses drastically exceeding the nocturnal levels are required to exert clear effects (Barrett *et al.*, 2010).

A rapidly developing area on melatonin research relates to its impact on the reproductive system. Several recent studies have shown that melatonin promotes the development of sheep, pigs, cattle, and mouse embryos in the *in vitro* situation with the potential mechanisms being related to its anti-oxidant and anti-apoptotic capacities (Ishizuka *et al.*, 2000; Abecia *et al.*, 2002; Papis *et al.*, 2007; Kang *et al.*, 2009). Melatonin also acts on the ovaries, with direct effects on granulosa cell steroidogenesis and follicular functions in mammals (Wang *et al.*, 2012; Chuffa *et al.*, 2013).

This work was undertaken in an attempt to determine whether melatonin can protect bovine mammary epithelial cells and quail ovary granulosa cells from lipopolysaccharide (LPS)-induced cell damage *in vitro*. Moreover, in order to check if the drastically exceeding physiological melatonin doses affect steroidogenesis, the effects of melatonin on progesterone secretion in granulosa cells of the Japanese quail

was also examined.

The first study (Chapter 2) attempted to test the hypothesis that melatonin would enable to decrease the grade of inflammation in bovine mammary epithelial cells induced by LPS *in vitro*. The Chapter 3 focuses on the effect of melatonin on LPS-stimulated cultured granulosa cells of the Japanese quail. To further test the effects of melatonin on progesterone production in granulosa cells, the basal progesterone secretion and the *in vivo* situation were investigated in the Chapter 4.

In the Chapter 5, the significance and possible application of the main findings of the present work to livestock production are discussed. Finally, the Chapter 6 summarizes the most important results obtained form Chapter 2 through 4.

Chapter 2

The anti-inflammatory and antioxidant effects of melatonin on LPS-stimulated bovine mammary epithelial cells

2.1 Introduction

Despite worldwide efforts to reduce its prevalence, mastitis remains a major economic threat to the dairy industry (Tothova *et al.*, 2014). It has been reported that the economic losses associated with clinical mastitis range from €61 to €97 per farmed cow per year, due to a combination of the reduction of milk output and the cost of controlling the disease (Hogeveen *et al.*, 2011). Mastitis is a complex disease. Its incidence is influenced by microorganisms, the surrounding environment, and the defense mechanism in the udder tissue (Ellah, 2013). Two distinct patterns in the epidemiology of mastitis have been reported. The first is a contagious disease pattern in which pathogenic microorganisms are transferred from animal to animal, and the second is a pattern of opportunistic microorganisms (Osman *et al.*, 2010). Inflammatory reactions accompanying mastitis cause severe damage to the mammary secretory epithelium, and as a consequence, reduce milk yield (Wellnitz *et al.*, 2006).

Gram-negative bacteria are the most frequent cause of mastitis (Fox, 2009). Lipopolysaccharide (LPS) released from gram-negative bacteria is considered to be an important stimulus of bovine mammary epithelial cell (bMEC) inflammation

(Schmitz *et al.*, 2004). LPS induces intracellular signaling via the cell surface receptor Toll-like receptor 4 (TLR4) in collaboration with other molecules, such as LPS-binding protein (LBP) and cluster of differentiation 14 (CD14) (Chow *et al.*, 1999; Su *et al.*, 2000). TLR engagement activates myeloid differentiation factor 88 (MyD88)-dependent and -independent intracellular signaling pathways (Bandow *et al.*, 2012). Both signaling pathways induce activation of nuclear factor-κB (NF-κB), resulting in the release of inflammatory cytokines and chemokines (Medzhitov and Kagan, 2006; Kawai and Akira, 2010). It has been reported that cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Alluwaimi, 2004; Sakemi *et al.*, 2011), and chemokines, such as chemokine CC motif ligand (CCL)2, CCL3, CCL5, CCL20, chemokine CXC motif ligand (CXCL)1, and CXCL2, play important roles in the pathogenesis of mastitis (Zheng *et al.*, 2006; Mount *et al.*, 2009; Roussel *et al.*, 2015). The levels of these cytokines and chemokines are increased in gram-negative bacteria-infected and LPS-infused mammary glands (Zheng *et al.*, 2006; Fu *et al.*, 2014). Elevated concentrations of chemokines induce the migration of inflammatory leukocytes into the infected area (Zlotnik and Yoshie, 2000), and cytokines amplify the inflammatory response by inducing the production of acute-phase proteins (APPs) such as serum amyloid A (SAA), haptoglobin, C-reactive protein (CRP), ceruloplasmin, α-1 antitrypsin, and fibrinogen (Hiss *et al.*, 2004; Eckersall *et al.*, 2011; Tothova *et al.*, 2014; Thomas *et al.*, 2015). Following the inflammatory stimulus, reactive oxygen species and reactive nitrogen species

(ROS/RNS) are produced and act in concert to induce cell damage (Reuter *et al.*, 2010).

Besides the multi-function of melatonin in both plants and animals mentioned in the Chapter 1, melatonin metabolites, including cyclic-3-hydroxymelatonin, N-acetyl-N-formyl-5-methoxykynuramine, and N-acetyl-5-methoxykynuramine, also have the ability to scavenge ROS and RNS (Galano *et al.*, 2013; Galano *et al.*, 2014). The reduction in free radical-mediated damage by melatonin thus contributes to its anti-inflammatory effects (Mauriz *et al.*, 2013). A number of studies attest to 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 (Lowes *et al.*, 2013), and also diminishes the levels of TNF-α, IL-1β, and oxidative stress mediators in different regions of rat brains after intracerebroventricular administration of LPS (Tyagi *et al.*, 2010). In pregnant mice, melatonin reduces LPS-induced increases in TNF-α levels in the maternal serum and fetal brain (Xu *et al.*, 2007). Melatonin also decreases TNF-α, IL-1β, IL-6 levels and reduces the number of apoptotic neurons after intraventricular Klebsiella pneumoniae injection in rats (Wu *et al.*, 2011). Finally, expression of *TNF-α*, *IL-1β*, *IL-6*, and *IL-8* mRNA in LPS-stimulated RAW264.7 cells is inhibited by melatonin treatment (Xia *et al.*, 2012).

All of these studies demonstrate the ability of melatonin to suppress pro-inflammatory cytokine levels and reduce oxidative stress in experimental inflammation. To my knowledge, however, there have been only two investigations

that have addressed the role of melatonin on mastitis model. Boulanger *et al.* (2002) evaluated the effect of melatonin and a proteinase inhibitor in bovine neutrophil-induced mammary cell damage. Recently, a Chinese research team reported the protective effect of melatonin on a mouse mastitis model (Shao *et al.*, 2015). Here, a series of experiments was conducted to determine whether melatonin can protect bMECs from LPS-induced cell damage. The present study investigated the effects of melatonin on the mRNA levels of pro- and anti-inflammatory cytokines, chemokines, and positive and negative APPs in LPS-stimulated bMECs. This study also examined ROS/RNS levels in the cell culture medium and evaluated the potential benefits of melatonin on the expression of anti- and pro-apoptotic regulators and cell viability following LPS treatment. Finally, this study investigated activation of the TLR4 and Nrf2 signaling pathways to uncover the molecular mechanisms underlying the protective function of melatonin in LPS-induced bMEC inflammation.

2.2 Materials and methods

2.2.1 Animals

All 12 lactating Holstein cows used in this study were handled in accordance with the regulations of Hiroshima University for animal experiments, and all experimental procedures were approved by the animal care committee of Hiroshima University.

2.2.2 Leukocyte isolation

Samples of 15 mL blood were collected by coccygeal venipuncture and immediately centrifuged at 1,800 × *g* for 15 min at 4°C. The leukocyte layer was transferred by pipette to a 1.5 mL centrifugation tube and re-centrifuged. The serum and erythrocytes were carefully removed by pipette and the leukocytes were collected.

2.2.3 Cell culture and treatment

The isolation and purification of epithelial cells from milk was performed as described previously (Wellnitz *et al.*, 2006). In brief, fresh milk from cows (800 mL/cow) with no clinical signs of mastitis and free of detectable bacteria in the milk (checked by the veterinary of the University farm) was shipped to the laboratory in a thermos flask (28–32 °C) within 15 min of collection. The milk sample was centrifuged in 50 mL tubes for 15 min at 1,800 × *g*. Cell pellets were resuspended in 12 mL Dulbecco's phosphate-buffered saline (DPBS; Nissui Pharmaceutical, Tokyo, Japan), and transferred to 15 mL tubes. The cells were washed three times by resuspension in DPBS and centrifugation for 5 min at 500 g. After washing, the cells from each cow were seeded in 5 mL DMEM/F12 medium (Gibco BRL/Invitrogen, Karlsruhe, Germany) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 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). Cells were cultured at 37°C in an atmosphere of 90% humidity and 5% CO_2. The medium was changed every 48 h. When cells reached confluence (~3

weeks), they were digested and propagated using 1 mL dissociation solution (CTK; ReproCELL, Yokohama, Japan). All experiments were performed with cells at the second passage.

A stock solution was prepared by dissolving 5 mg of melatonin (Sigma-Aldrich) in 100 µL ethanol. The working concentrations were prepared by dilution in cell culture medium to give final concentrations of 10 and 100 µg/mL. As controls, cells were incubated with ethanol at the highest concentration used for the melatonin treatment. Cells were pre-incubated with or without melatonin for 12 h and then 100 ng/mL LPS (from *E. coli*; ALX-581-014-L001; Enzo Life Sciences, Farmingdale, NY, USA), a concentration determined by the trial test (Figure 2.2), was added to the culture medium stimulating for another 12 h.

2.2.4 RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using NucleoSpin RNA (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The total RNA was treated with DNase I at 18–25 °C for 15 min. RNA concentrations were calculated from the optical density at 260 nm, assuming an OD260 unit is equivalent to 40 µg/mL RNA. The RNA purity was determined by measuring the absorbance ratio at 260/280 nm. Only samples with a ratio > 1.8 were used. Aliquots of 240 ng of total RNA were reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan). To confirm the purity of the cells, RT-PCR was performed on total RNA from leukocytes and bMECs using

primers specific for *casein α-S2 (CSN1S2)* (Forward: 5′-TCAGCCAGCG TTACCAGAAAT-3′. Reverse: 5′-AACATGCTGGTTGTATGAAGTAAAGTG-3′), a MEC specific gene; and for *cluster of differentiation 45 (CD45)* (Forward: 5′-ATGTATTTGTGGCTTAAAC-3′. Reverse: 5′-TTCTCATCATCCCTTTCAAGC-3′) (Ballingall *et al.*, 2001), a leukocyte specific gene. RT-PCR was performed using TaKaRa Ex Taq (Takara Bio, Shiga, Japan) on an Applied Biosystems GeneAmp PCR System 9700 (Life Technologies, Darmstadt, Germany). The RT-PCR mixture consisted of 0.25 μL TaKaRa Ex Taq, 5 μL 10× Ex Taq buffer, 4 μL dNTP mixture, 0.5 μM forward and reverse primers, 1 μL template, and ddH_2O to a total volume of 50 μL. The reaction procedure was as follows: initial denaturation at 98°C for 2 min and then 50 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 7 min. cDNA products were resolved on 2% (w/v) agarose gels.

qRT-PCR was performed using SYBR Premix Ex Taq II (Takara Bio) on an Applied Biosystems StepOne Real-Time PCR System (Life Technologies) according to the method described previously (Guangmin *et al.*, 2015). In brief, the qPCR mixture consisted of 10 μL SYBR Premix Ex Taq II, 0.4 μM forward and reverse primers, 0.4 μL ROX Reference Dye, 2 μL template, and ddH_2O to a total volume of 20 μL. The amplification parameters were as follows: initial denaturation at 95°C for 30 s followed by 50 cycles of denaturation at 95°C for 5 s, annealing and extension at 60–68°C for 34 s, and a melting curve from 60 to 95°C, increasing in increments of 0.5°C every 5 s. Normalization was performed using the housekeeping gene

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. Primer sequences are listed in Table 2.1. Relative mRNA expression was calculated by the $2^{-\Delta\Delta ct}$ method. Samples from 5 cows were measured in duplicate.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

Cell culture medium was pre-cleared by centrifugation at 3,000 × *g* for 20 min to remove cells and then stored at −20°C until use. To evaluate the oxidative stress level, dityrosine in duplicate samples of culture medium were measured using a competitive ELISA kit (JaICA, Nikken SEIL, Shizuoka, Japan), according to the manufacturer's instructions. Dityrosine concentrations were calculated by comparison with a standard curve that typically ranged from 0.05 to 12 μM.

2.2.6 The Griess reaction

Levels of the nitric oxide (NO) metabolite nitrite in samples of cell-free culture medium were measured with a nitrite colorimetric assay kit using the Griess reaction (Dojindo Molecular Technologies, Tokyo, Japan), according to the manufacturer's instructions. Samples were measured spectrophotometrically (Model 680 microplate reader S/N 22002; Bio-Rad Laboratories, Hercules, CA, USA) and nitrite concentrations (μM) were calculated with reference to a standard curve. Measurements were performed in duplicate.

2.2.7 Fluorescence microscopy

Cell viability was determined using a Fluo Cell Double Staining Kit (MoBiTec, Göttingen, Germany). bMECs were placed on a coverslip and incubated for 15 min at 37°C in the presence of calcein-AM (0.4 μM) and propidium iodide (PI, 0.3 μM). The coverslip was placed on a slide and the cells were examined with a Keyence BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Digital images were captured at 100 × magnification.

2.2.8 Cell viability analysis

The viability of bMECs was quantified using an MTT assay. Briefly, cells were seeded in 96-well plates at 1×10^5 cells/well and incubated with or without melatonin (10 or 100 μg/mL) for 12 h. Medium or 100 ng/mL LPS was then added and the cells were incubated for a further 12 h. The cells were washed three times with DPBS, and 5 mg/mL MTT (Sigma-Aldrich) in DPBS was added to each well. After 4 h incubation at 37°C, the supernatant was removed and 100 μL dimethyl sulfoxide (Nacalai Tesque) was added to stop the reaction. The plates were shaken for 15 min, and the absorbance at 570 nm was measured using a spectrophotometer (Model 680 microplate reader S/N 22002; Bio-Rad Laboratories). The measurements were performed in sextuplicate.

2.2.9 Statistical analysis

Continuous variables are expressed as the mean ± standard deviation (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). $P<0.05$ was considered statistically significant.

Table 2.1. Primers used for qRT-PCR.

Genes	Primer sequence (5'–3')	Product size (bp)	Tm (°C)
GADPH	Forward: GTCTTCACTACCATGGAGAAGG	199	60
	Reverse: TCATGGATGACCTTGGCCAG		
LBP	Forward: GGTGCGCAAGAGGATACTGA	192	60
	Reverse: AAGAGATTCAGCAGCCACCC		
CD14	Forward: GACCTCCGCTGTCTTTCCAG	188	60
	Reverse: CTCGACGGCAACCATACACT		
TLR4	Forward: TCCCCGACAACATCCCCATA	159	64
	Reverse: GGCCCTGAAATGTGTCGTCT		
NF-κB	Forward: CAGCCTGGTGGGAAAACACT	150	60
	Reverse: CAGGCATCTGTCATTCGTGC		
TNF-α	Forward: CCACGTTGTAGCCGACATC	156	60
	Reverse: CCCTGAAGAGGACCTGTGAG		
IL-1β	Forward: CAGTGCCTACGCACATGTCT	174	60
	Reverse: GCCAGCACCAGGGATTTTTG		
IL-6	Forward: GCTGAATCTTCCAAAAATGGAGG	200	60
	Reverse: GCTTCAGGATCTGGATCAGTG		
IL-8	Forward: ATGACTTCCAAGCTGGCTGTTG	149	60
	Reverse: TTGATAAATTTGGGGTGGAAAG		
GM-CSF	Forward: GACTCCCAGGAACCAACGTG	113	60
	Reverse: TCGTAGTGGGTGGCCATCAT		
IL-1Ra	Forward: CTCGAGGTCACAGGATGGACA	136	60
	Reverse: ACATCCCAGATCCTGAAGGC		
CCL2	Forward: TTAACTCCCAAGTCGCCTGC	158	68
	Reverse: CTGCTTGGGGTCTGCACATA		
CCL3	Forward: TGCACTGACGCTCAAGCC	181	60
	Reverse: CGATTTTGCGAGAAAGCTGCC		
CCL5	Forward: GCCTTGAACCTGAACTTGCG	112	64
	Reverse: TGGAATCTGTGCCTTCCCAG		
CCL20	Forward: CAGCAAGTCAGAAGCAAGCA	169	60
	Reverse: CTTTGGATCTGCACACACAGC		
CXCL1	Forward: CCGCCCCCATGGTTAAGAAA	161	60
	Reverse: AAACACAGTCCAGATGGCCC		
CXCL2	Forward: CCAGCTCTAACTGACCAGGTG	116	60
	Reverse: ATGGCCTTAGGAGGTGGTGA		
SAA	Forward: CCTGGGCTGCTAAAGTGATC	184	60
	Reverse: TACTTGTCAGGCAGGCCAG		
haptoglobin	Forward: GCATGCTGGAAATGGGGTGT	141	60
	Reverse: ACCAAGTACTCCACGTAGCC		
ceruloplasmin	Forward: TGACCTCTTCCCTGGGACAT	147	60
	Reverse: CCAGACTTGATCTCTTCGTTTGG		
CRP	Forward: TTGGTGGCACAGACTGACC	176	66

	Reverse: TGAAGGCTTTGAGTGGTGTCC		
α-1 antitrypsin	Forward: GACTTCCATGTGGACGAGCA	122	68
	Reverse: TTGCCCACGTAGTCCAACAG		
fibrinogen	Forward: AAACCGGACCATGACCATCC	176	60
	Reverse: ACGCTCCACCCCAGTAGTAT		
Nrf2	Forward: CATGGCATCACCAGACCACT	130	60
	Reverse: CGGTGTTTTGGGACCCTTCT		
HO-1	Forward: CAAGCGCTATGTTCAGCGAC	198	60
	Reverse: TTGGTGGCACTGGCGATATT		
iNOS	Forward: CTTGTTCTCGAGGTGCCCAT	174	60
	Reverse: GTCCCGGACTCCAACTTCTG		
Bcl-2	Forward: TGGAGGAGCTCTTCAGGGAC	140	64
	Reverse: GTACTCGGTCATCCACAGGG		
Bax	Forward: GCGCATCGGAGATGAATTGG	153	60
	Reverse: TAGAAAAGGGCGACAACCCG		

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *LBP*, lipopolysaccharide binding protein; *CD14*, cluster of differentiation 14; *TLR4*, Toll-like receptor 4; *NF-κB*, nuclear factor κB; *TNF-α*, tumor necrosis factor-α; *IL*, interleukin; *IL-1Ra*, IL-1 receptor antagonist; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *CCL*, chemokine CC motif ligand; *CXCL*, chemokine CXC motif ligand; *SAA*, serum amyloid A; *CRP*, C-reactive protein; *Nrf2*, nuclear factor E2-related factor; *HO-1*, heme oxygenase 1; *iNOS*, inducible nitric oxide synthase; *Bcl-2*, B-cell lymphoma 2; *Bax*, Bcl-2-associated X protein.

2.3 Results

2.3.1 Morphology and purity of bMECs

The morphology of bMECs is shown in Figure 2.1A. The purity of the epithelial cells was examined by analysis of *CSN1S2 and CD45* mRNA expression. The epithelial cell morphology, the expression of *CSN1S2* and the absence of *CD45* (Figure 2.1B) confirmed the cell purity.

2.3.2 The effect of different LPS concentrations on inflammatory factors

In the trial test, both 100 and 1,000 ng/mL LPS significantly increased the expression of *TNF-α* and *SAA* mRNA in bMECs (Figure 2.2). However, there was no significant difference between the 100 and 1,000 ng/mL LPS groups. Thus, a series of experiments were conducted using 100 ng/mL LPS.

2.3.3 Melatonin inhibits the TLR4 signaling pathway in LPS-stimulated bMECs

As shown in Figure 2.3, 100 ng/mL LPS significantly increased the expression of *LBP*, *CD14*, *TLR4*, and *NF-κB* mRNA in bMECs. Melatonin markedly suppressed the expression of *LBP* mRNA at both concentrations tested (100 ng/mL LPS + 10 or 100 μg/mL melatonin, $P < 0.05$; Figure 2.3A). *CD14* mRNA levels were not significantly different in the control (LPS 0 ng/mL + melatonin 0 μg/mL) group and the 100 ng/mL LPS + 10 or 100 μg/mL melatonin groups (Figure 2.3B). However, melatonin had no significant effect on the LPS-stimulated increase in *TLR4* and *NF-κB* mRNA (Figures 2.3C and 2.3D).

2.3.4 Melatonin modulates LPS-induced cytokine expression

LPS caused substantial increases in the mRNA levels of the pro-inflammatory cytokines *TNF-α, IL-1β*, *IL-6*, *IL-8*, and *GM-CSF* (Figure 2.4). Addition of 10 μg/mL melatonin significantly decreased *TNF-α*, *IL-6*, and *GM-CSF* mRNA levels ($P < 0.05$, Figures 2.4A, 2.4C and 2.4E), whereas *IL-1β* mRNA levels were significantly decreased by melatonin at concentrations of 10 and 100 μg/mL ($P < 0.05$, Figure 2.4B). However, melatonin had no significant effect on *IL-8* mRNA levels in LPS-stimulated cells (Figure 2.4D). With regard to the anti-inflammatory cytokine *IL-1Ra*, although LPS treatment markedly decreased *IL-1Ra* mRNA level, pretreatment with 100 μg/mL melatonin rescued its expression (Figure 2.4F).

2.3.5 Melatonin inhibits LPS-induced chemokine expression

LPS stimulation substantially increased the mRNA levels of *CCL2*, *CCL3*, *CCL5*, *CCL20*, *CXCL1*, and *CXCL2* (Figure 2.5). Melatonin at both 10 and 100 μg/mL markedly suppressed the expression of *CCL2* mRNA ($P < 0.05$, Figure 2.5A) and 10 μg/mL significantly decreased *CCL5* mRNA level ($P < 0.05$, Figure 2.5C). However, melatonin had no significant effect on the LPS-induced increase in *CCL3*, *CCL20*, *CXCL1*, and *CXCL2* mRNA (Figures 2.5B, 2.5D, 2.5E and 2.5F).

2.3.6 Melatonin modulates the LPS-induced expression of acute-phase protein

LPS treatment clearly increased the expression of *SAA*, *haptoglobin*,

ceruloplasmin, *CRP*, and α-1 *antitrypsin* mRNA (Figure 2.6). When present at 10 μg/mL, melatonin significantly decreased *CRP* mRNA level ($P < 0.05$, Figure 2.6D), and at 100 μg/mL, it markedly attenuated the increase in *SAA*, *ceruloplasmin*, and *α-1 antitrypsin* mRNA levels ($P < 0.05$, Figures 2.6A, 2.6C, and 2.6E). In addition, the LPS-induced increase in *haptoglobin* mRNA was significantly inhibited by melatonin at concentrations of 10 and 100 μg/mL ($P < 0.05$, Figure 2.6B). In contrast, *fibrinogen* mRNA level was markedly suppressed by LPS, and this was partially rescued by pretreatment with 100 μg/mL melatonin ($P < 0.05$, Figure 2.6F).

2.3.7 Melatonin activates the Nrf2 antioxidant defense pathway in LPS-stimulated bMECs

As shown in Figure 2.7, LPS significantly inhibited the expression of *Nrf2* and *heme oxygenase-1* (*HO-1*) mRNA in bMECs. Melatonin concentrations of 10 and 100 μg/mL rescued *Nrf2* mRNA expression (Figure 2.7A), while 100 μg/mL melatonin rescued the expression of *HO-1* (Figure 2.7B). Finally, the level of *inducible NO synthase* (*iNOS*) mRNA was substantially increased by LPS stimulation; however, melatonin pretreatment had no significant effect (Figure 2.7C).

2.3.8 Melatonin inhibits oxidative stress in LPS-stimulated bMECs

To examine oxidative stress, dityrosine and nitrite levels in the supernatants of treated bMECs were measured. ELISA assays showed that LPS substantially decreased the production of dityrosine ($P < 0.05$), but this effect was inhibited by

pretreatment with 100 μM melatonin (Figure 2.8A). The nitrite level in the culture medium, determined by the Griess reaction, was markedly increased by LPS stimulation (Figure 2.8B, $P < 0.05$). As expected, melatonin significantly suppressed the nitrite levels at both 10 and 100 μg/mL ($P < 0.05$).

2.3.9 Melatonin prevents LPS-induced cell death

The results of qPCR show that LPS stimulation markedly attenuated the expression of *Bcl-2* mRNA ($P < 0.05$), but pretreatment with 10 μg/mL melatonin was sufficient to inhibit the effect (Figure 2.9A). However, while the expression of *Bax* mRNA was significantly increased by LPS stimulation ($P < 0.05$), there was no significant difference in its expression in cells pre-incubated with or without melatonin (Figure 2.9B). The viability of bMECs were examined by co-incubating them with calcein-AM and PI, two fluorescent dyes that label live and dead cells, respectively. The result showed that although LPS reduced the cell viability, melatonin at concentrations of 10 and 100 μg/mL prevented cell death (Figure 2.10A). Similarly, quantification of cell viability using the MTT assay revealed that 100 μg/mL melatonin was able to prevent the loss of cell viability caused by LPS stimulation (Figure 2.10B).

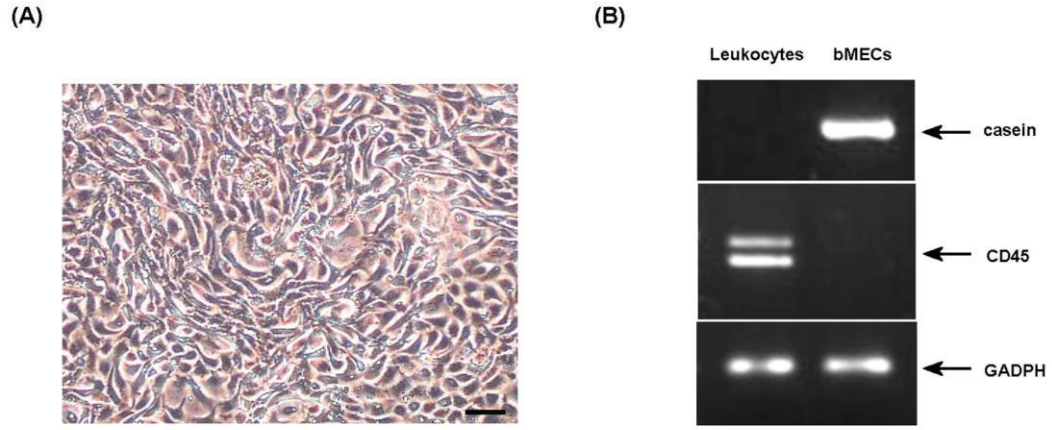

Figure 2.1. Characteristics of purified bMECs. (A) Photomicrograph showing the morphology of cultured bMECs. Scale bar: 10 μm. (B) RT-PCR analysis showing that bMECs are positive for *CSN1S2*, but negative for *CD45* expression.

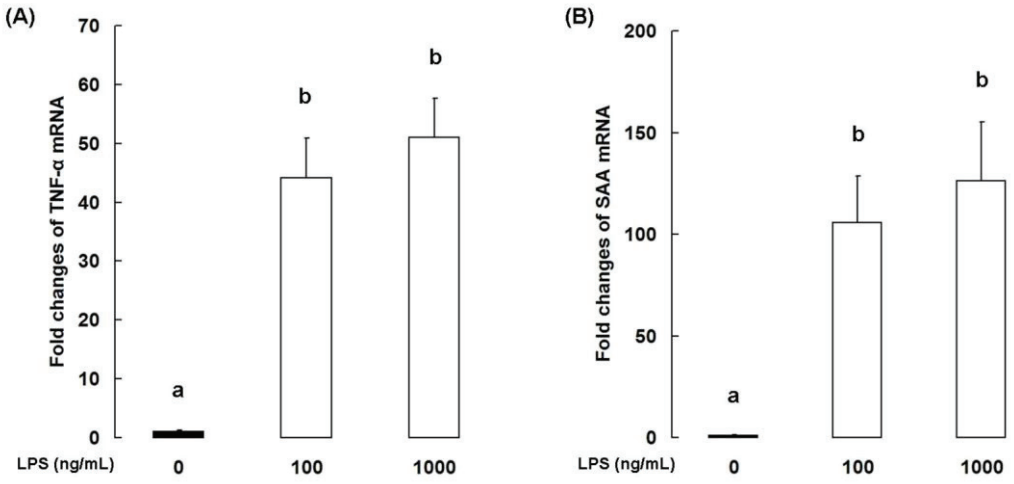

Figure 2.2. Both 100 and 1,000 ng/mL LPS increase the expression of *TNF-α* and *SAA* mRNA in bMECs. qPCR analysis of (A) *TNF-α*, and (B) *SAA*. Data are the mean ± SD of three independent experiments. Values with different superscripts are significantly different at $P<0.05$.

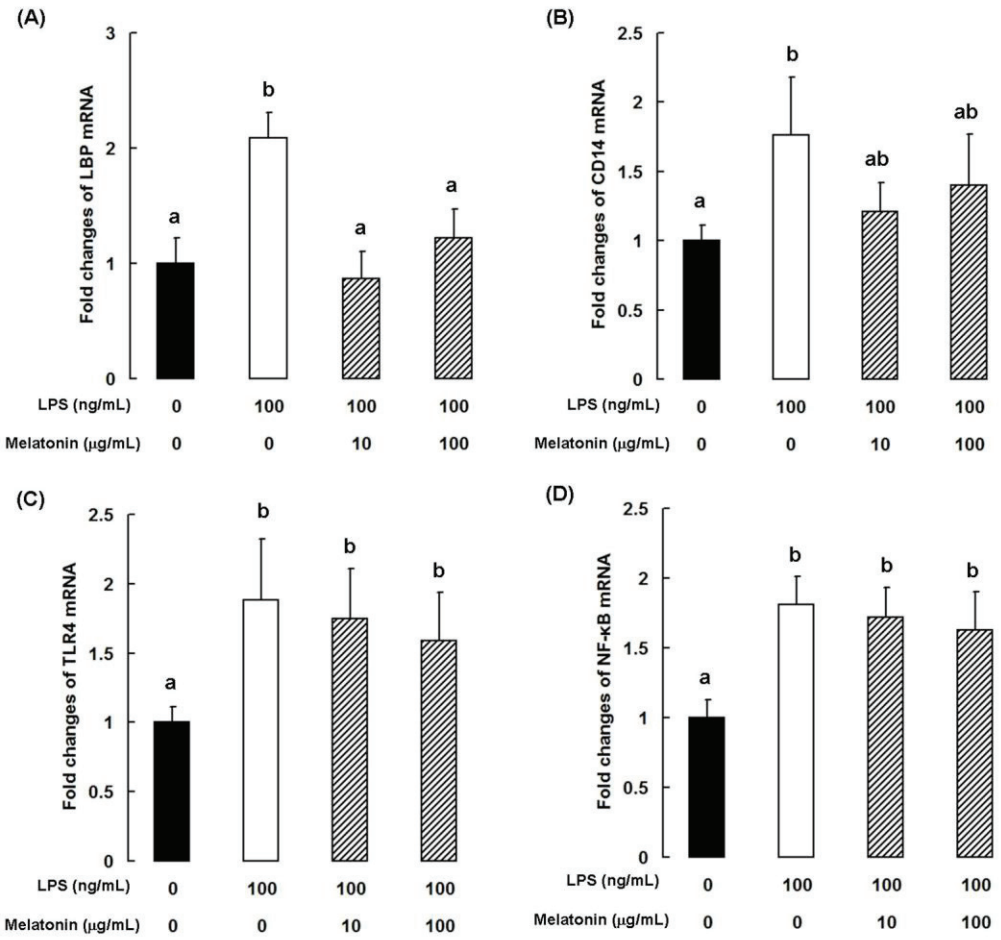

Figure 2.3. Melatonin inhibits the TLR4 signaling pathway in LPS-stimulated bMECs. qPCR analysis of (A) *TLR4*, (B) *LBP*, (C) *CD14*, and (D) *NF-κB*. Data are the mean ± SD of five independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

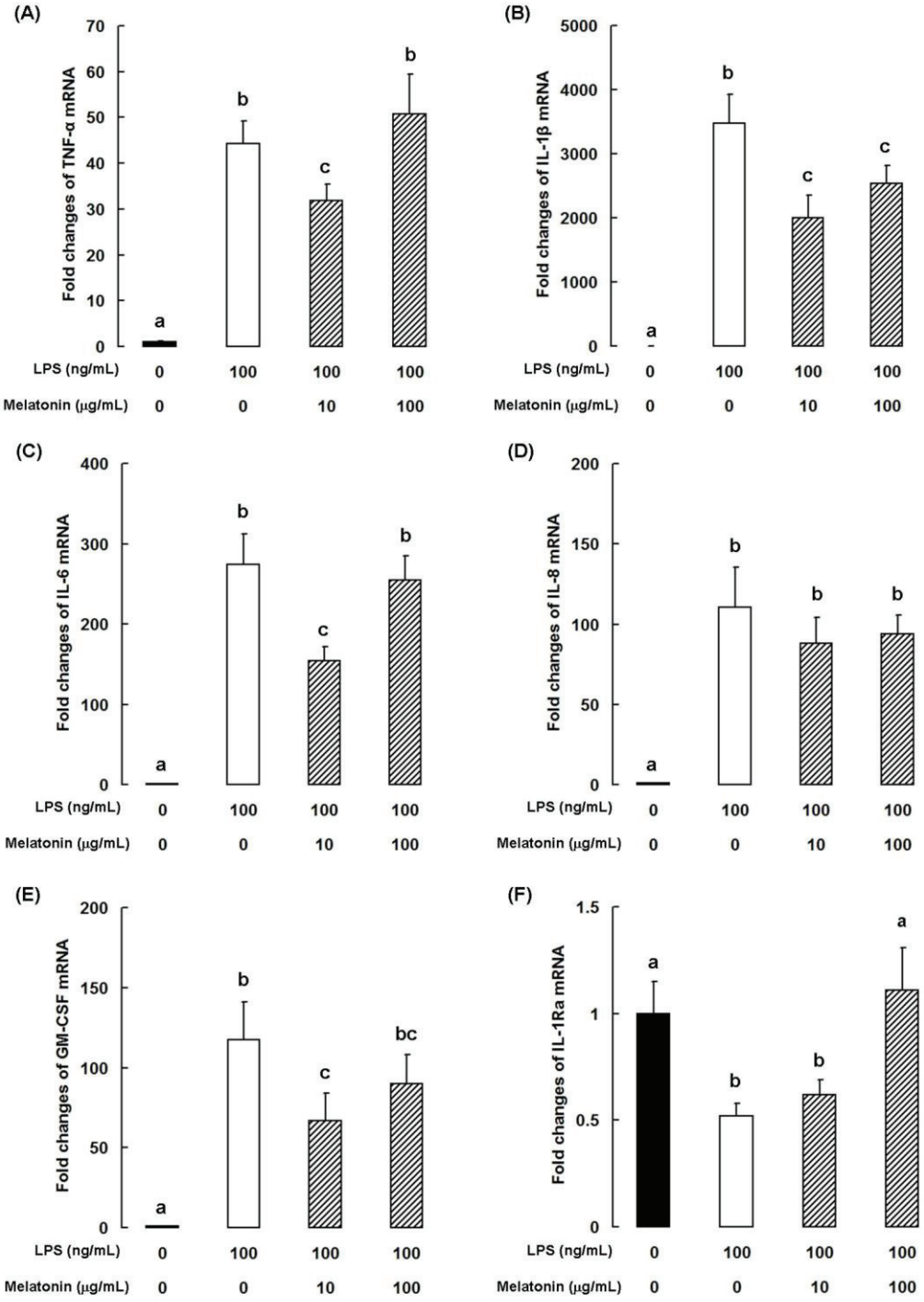

Figure 2.4. Melatonin modulates LPS-induced cytokine expression in bMECs. qPCR analysis of (A) *TNF-α*, (B) *IL-1β*, (C) *IL-6*, (D) *IL-8*, (E) *GM-CSF*, and (F) *IL-1Ra*. Data are the mean ± SD of five independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

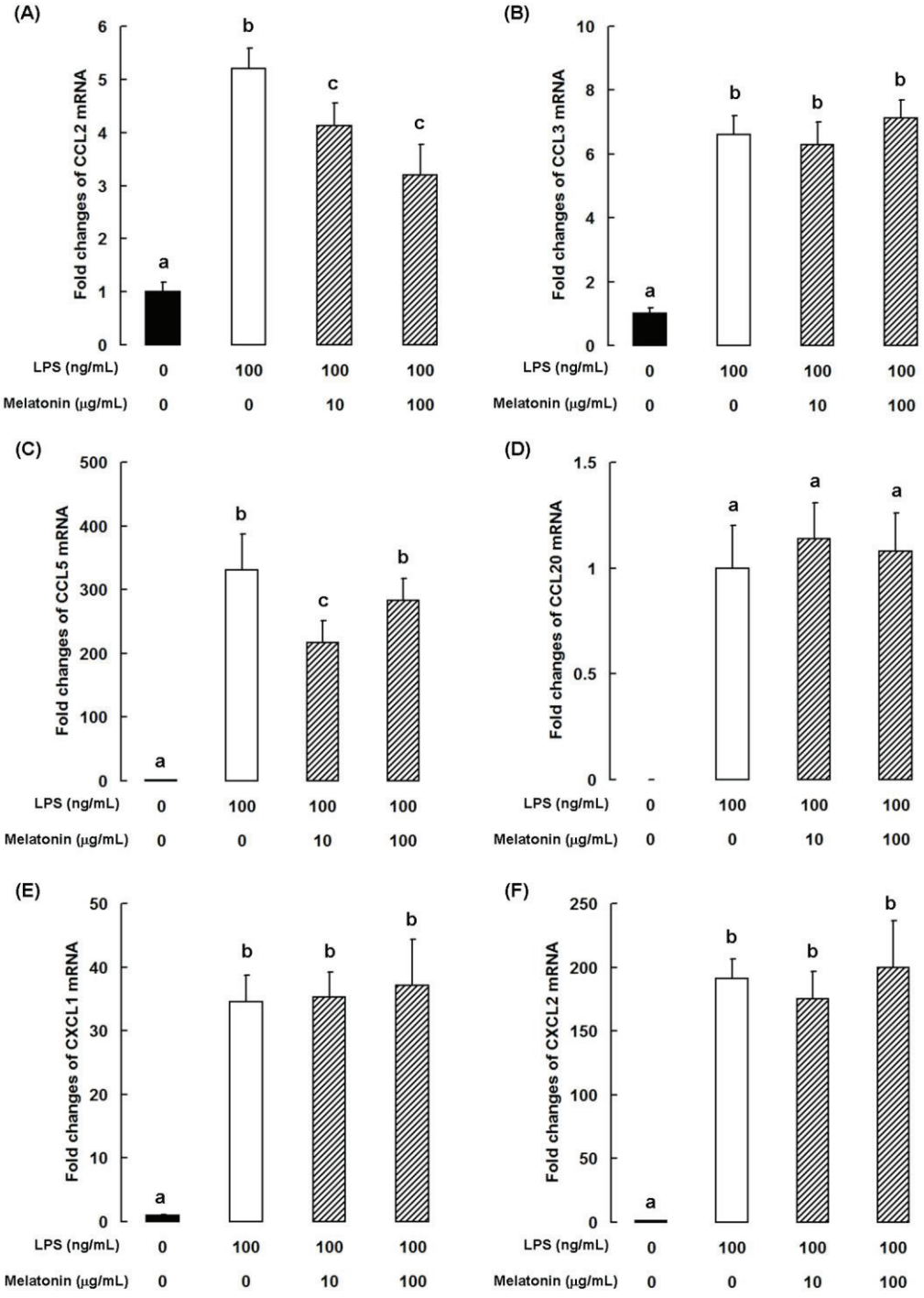

Figure 2.5. Melatonin inhibits LPS-induced chemokine expression in bMECs. qPCR analysis of (A) *CCL2*, (B) *CCL3*, (C) *CCL5*, (D) *CCL20*, (E) *CXCL1*, and (F) *CXCL2*. Data are the mean ± SD of five independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

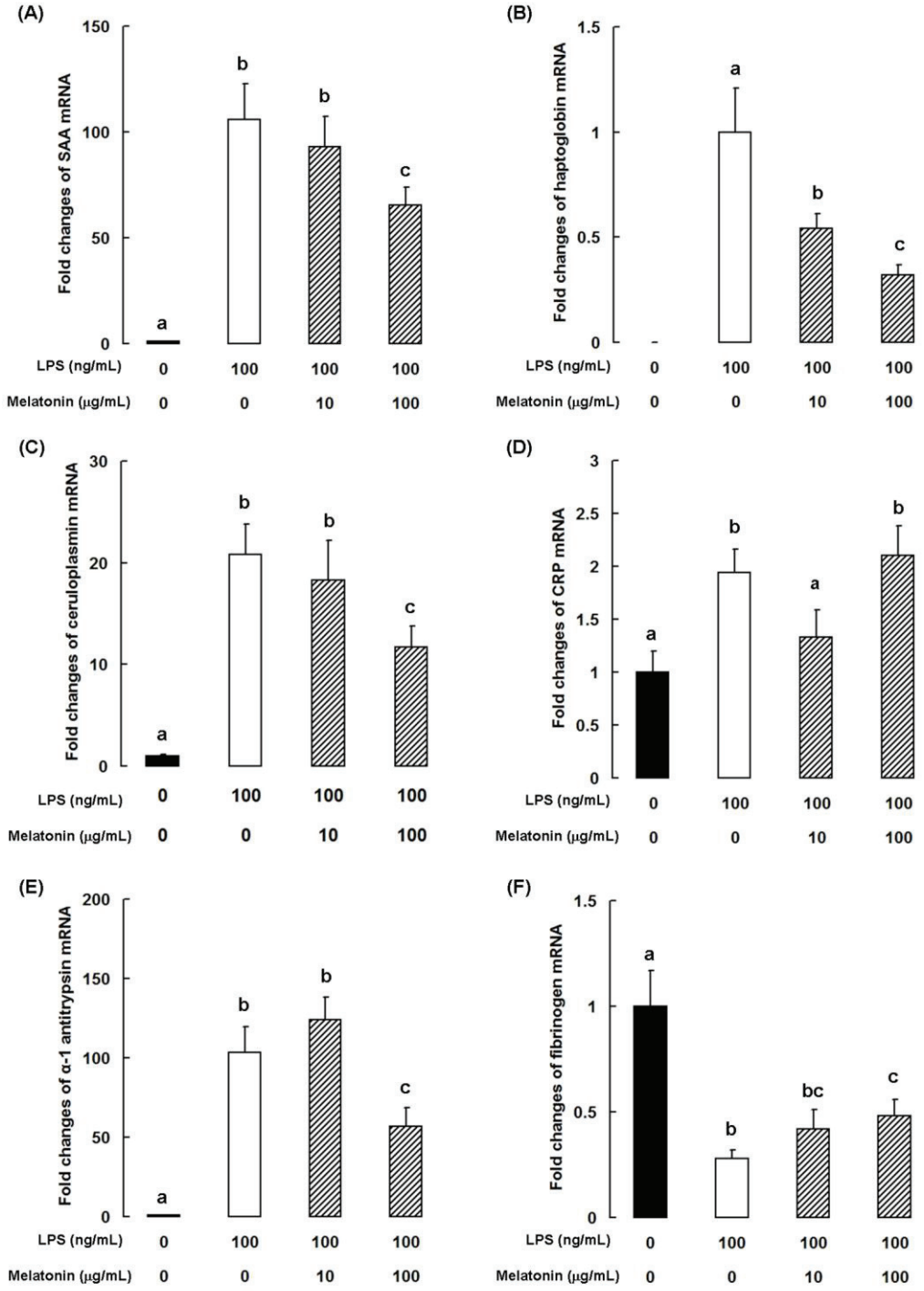

Figure 2.6. Melatonin modulates LPS-induced acute-phase protein expression in bMECs. qPCR analysis of (A) *SAA*, (B) *haptoglobin*, (C) *ceruloplasmin*, (D) *CRP*, (E) *α-1 antitrypsin*, and (F) *fibrinogen*. Data are the mean ± SD of five independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

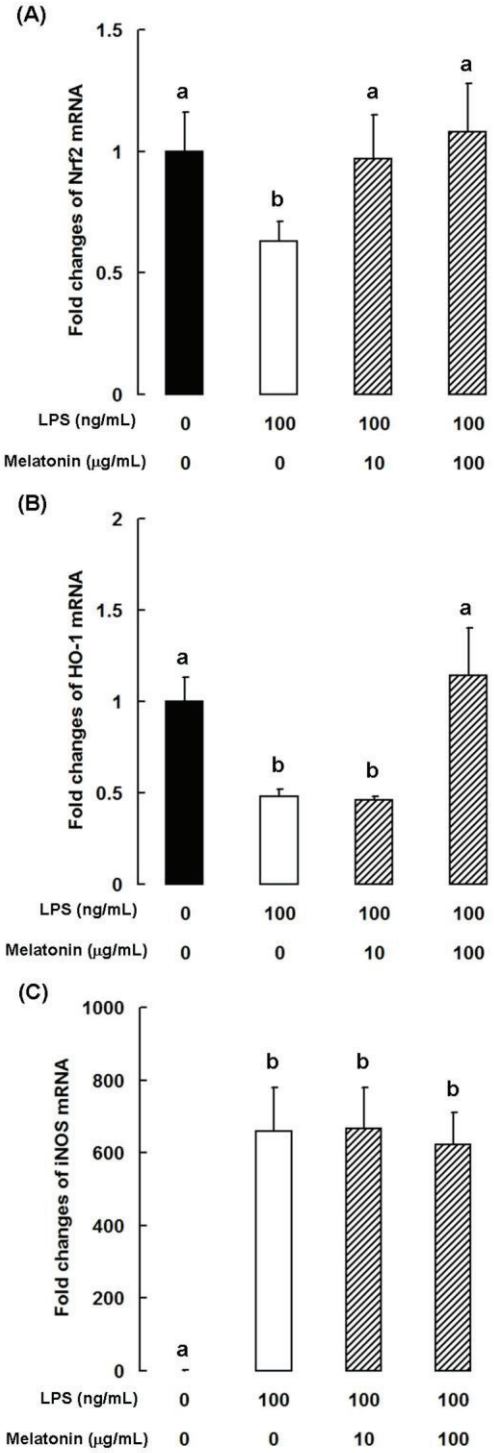

Figure 2.7. Melatonin activates the Nrf2 antioxidant defense pathway in LPS-stimulated bMECs. qPCR analysis of (A) *Nrf2*, (B) *HO-1*, and (C) *iNOS*. Data are the mean ± SD of five independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

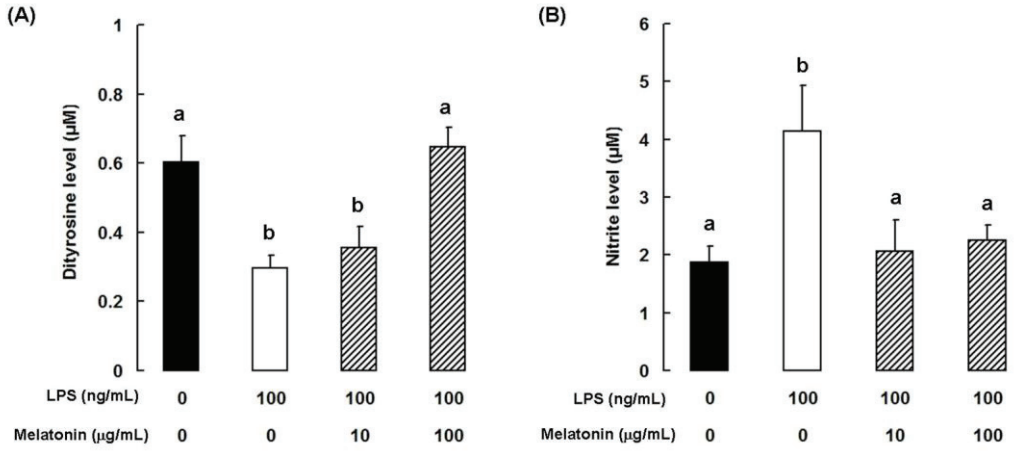

Figure 2.8. Melatonin reduces oxidative stress in LPS-stimulated bMECs. (A) dityrosine level and (B) nitrite level in the culture medium. Data are the mean ± SD of six independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

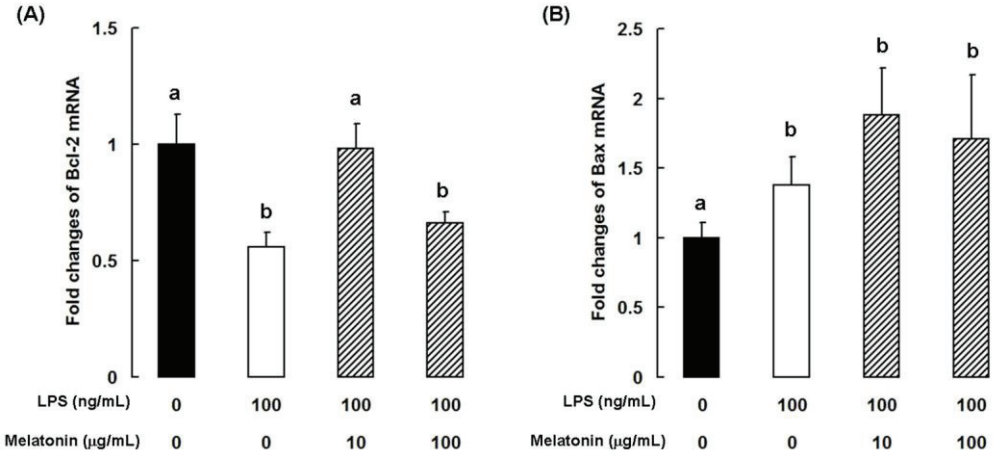

Figure 2.9. Melatonin modulates the expression of apoptosis regulatory genes in LPS-stimulated bMECs. qPCR analysis of (A) *Bcl-2* and (B) *Bax*. Data are the mean ± SD of five independent experiments, each performed in duplicate. Values with different superscripts are significantly different at $P<0.05$.

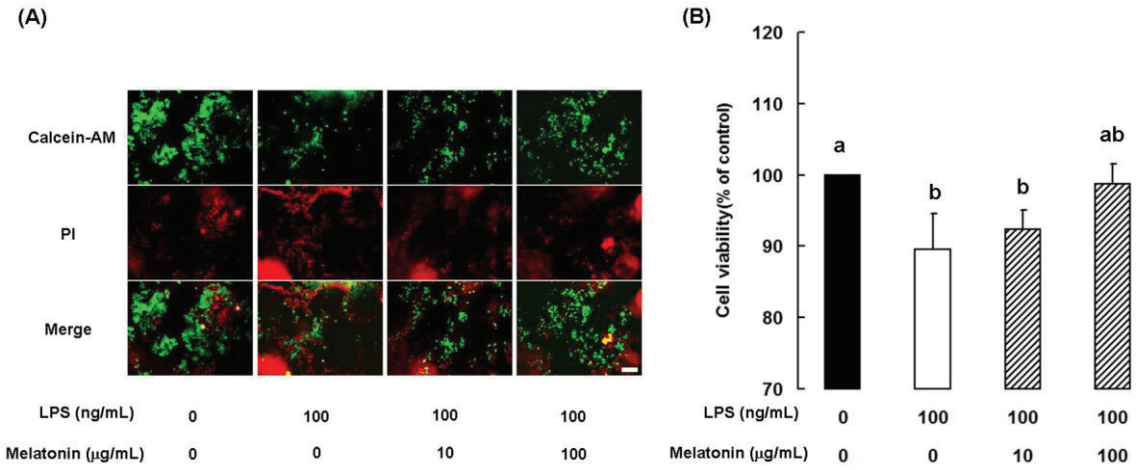

Figure 2.10. Melatonin prevents LPS-induced death of cultured bMECs. (A) Fluorescence microscopic images of cell double-staining. Data are representative of three independent experiments. Scale bar: 20 μm. (B) cell viability analyzed by MTT assay. Data are the mean ± SD of three independent experiments, each performed in sextuplicate. Values with different superscripts are significantly different at $P<0.05$.

2.4 Discussion

Mastitis is the most prevalent disease of dairy cattle worldwide and not only causes huge economic losses in the dairy industry but also threatens public health (Hogeveen *et al.*, 2011). The extensive use of antibiotics in dairy herds, however, leads to milk being discarded and increases consumer concern over food safety (Pieterse and Todorov, 2010; Yu *et al.*, 2016). At the same time, accompanied with the world population growing, more and better-quality food will be needed (Yu and Maeda, 2017). Accumulating evidence attributes the anti-inflammatory effects of melatonin to its direct antioxidant actions. However, the exact mechanism by which melatonin protects against mastitis and the key parameters that it influences remain to be elucidated. The present study confirmed that melatonin inhibits the TLR4 signaling pathway and diminishes mRNA expression of pro-inflammatory cytokines, chemokines, and positive APPs, but increases mRNA expression of an anti-inflammatory cytokine and a negative APP in LPS-stimulated bMECs. In addition, results showed that melatonin inhibits oxidative stress through activation of the Nrf2 antioxidant defense pathway and improves cell viability.

bMECs play an important role in protecting the mammary gland from severe inflammation caused by invading pathogens (Wellnitz *et al.*, 2006). After stimulation by molecules released from microorganisms, bMECs produce several immunomodulators that initiate the immune response and activate leukocytes in the udder (Pareek *et al.*, 2005). In the present study, the bMECs were isolated from fresh milk, a non-invasive method of collection. Cells from individual cows were obtained

and cultured separately to provide biological replicates. To ensure that the epithelial cells were pure, the expression of *CSN1S2* and the absence of the leukocyte specific gene CD45 were confirmed. As shown in Figure 2.1, the morphology and absence of CD45 suggest that the bMECs were not contaminated with leukocytes.

The application of melatonin to treat inflammation derives from previous researches where melatonin doses drastically exceeding the nocturnal levels (the peak of physiological concentration of melatonin is 20 ng/mL in bovine serum and milk [Eriksson et al., 1998]) are required to exert clear effects (Barrett *et al.*, 2010). There have been only two investigations that have addressed the role of melatonin on mastitis model before this study. In contrast to the present study, Boulanger *et al.* (2002) used a mammary epithelial cell line to evaluate 1,000 ng/mL LPS-induced oxidative stress response. It is a pretty important work showing the effect of melatonin and catalase on bovine neutrophil-induced mammary epithelial cell damage in a co-culture of bovine neutrophils and MAC-T cells. The anti-inflammatory effect of melatonin is reported on a mouse mastitis model (Shao *et al.*, 2015). Melatonin attenuated LPS-induced mammary histopathologic changes and myeloperoxidase activity in that model. Melatonin also inhibited LPS-induced inflammatory cytokines TNF-α, IL-1β and IL-6 production in mouse mammary tissues. *In vitro*, melatonin was found to inhibit 100 ng/mL LPS-induced inflammatory responses by activating peroxisome proliferator-activated receptor-γ in mouse MECs. Prior to the present study, a trial test was conducted, which reveals both 100 and 1,000 ng/mL LPS significantly increased the expression of *TNF-α* and *SAA* mRNA in bMECs, but there

was no significant difference between 100 and 1,000 ng/mL LPS groups (Figure 2.2). Thus, a series of experiments were conducted to demonstrate that melatonin protect bMECs from 100 ng/mL LPS-induced inflammatory and oxidative damage. The findings are consistent with previous observation that modulation of apoptosis requires high melatonin doses (Radogna *et al.*, 2007; Radogna *et al.*, 2009). The two investigations mentioned above also show that melatonin at 5.8–116 μg/mL effectively inhibited the LPS-induced mammary epithelial cell damage.

Cytokine are a broad category of small, nonstructural, secreted proteins that are synthesized by and induce a response in nearly all nucleated cells (Dinarello, 2000). They are primarily involved in immune and inflammatory responses, including responses to infection, trauma, and cancer, and also play roles in reproduction. Cytokines can be classified according to their biological activities. Pro-inflammatory cytokines, such as *TNF-α*, *IL-1β*, *IL-6*, *IL-8*, and *GM-CSF* studied here, generally exacerbate disease and infections, while anti-inflammatory cytokines, such as *IL-1Ra*, reduce inflammation and promote healing (Opal and DePalo, 2000). Considerable attention has focused on blocking the action of pro-inflammatory cytokines, particularly during overwhelming infection. Previous studies have shown that melatonin suppresses *TNF-α*, *IL-1β*, *IL-6*, and *IL-8* during bacterial infections or LPS treatment *in vitro* and *in vivo* (Xu *et al.*, 2007; Tyagi *et al.*, 2010; Wu *et al.*, 2011; Xia *et al.*, 2012; Lowes *et al.*, 2013; Shao *et al.*, 2015). The results of the present study suggest that melatonin decreases expression of *TNF-α*, *IL-1β*, *IL-6*, and *GM-CSF* mRNA in LPS-stimulated bMECs. This is the first demonstration of an inhibitory

effect of melatonin on *GM-CSF* expression. This study also reports for the first time that LPS-mediated suppression of *IL-1Ra* can be reversed by melatonin, which is likely to have a beneficial effect by reducing inflammatory responses.

Chemokines are chemotactic cytokines that induce leukocyte migration by interacting with G protein-coupled receptors. Chemokines play fundamental roles in the development, homeostasis, and function of the immune system (Zlotnik and Yoshie, 2012). During infection, microbial antigens initiating inflammatory responses provoke the production of numerous chemokines by local cells expressing TLRs. Effector leukocytes in the blood are attracted by chemokines and travel along the increasing chemokine concentration gradient towards the site of infection (Moser *et al.*, 2004). Earlier work has described only the effect of melatonin on *CXCL8* (also known as *IL-8*) and *CXCL10* (Jung *et al.*, 2010; Gitto *et al.*, 2012; Kang and Lee, 2012; Xia *et al.*, 2012). The present study is the first to examine the effect of melatonin on *CCL2*, *CCL3*, *CCL5*, *CCL20*, *CXCL1*, and *CXCL2* mRNA expression in LPS-stimulated bMECs. Results show that only *CCL2* and *CCL5* were suppressed. An understanding of the effect of melatonin on chemokines should help to define future directions in chemokine-based anti-inflammatory therapies. It is well recognized that the TLR4 signaling pathway is modulated by melatonin under inflammatory conditions (Kang and Lee, 2012; Xia *et al.*, 2012; Nduhiraband *et al.*, 2016). In the present study, melatonin reduced the transcription of *LBP* and *CD14*, which collaborate with TLR4 on the cell surface to activate intracellular inflammatory signaling pathways.

The acute-phase response is a series of non-specific and complex reactions occurring soon after the onset of stress, injury, trauma, infection, inflammation, and neoplasia, which aim to eliminate the infectious agents, restore homeostasis, and promote healing (Cray *et al.*, 2012; Tothova *et al.*, 2014). It is a highly coordinated process comprising a wide variety of behavioral, physiological, biochemical, and nutritional changes (Ceciliani *et al.*, 2012). The most important metabolic change is the significant increase or decrease in production of APPs (positive and negative APPs, respectively) (Murata *et al.*, 2004). The marked changes in APP levels that occur during infection and inflammation allow them to serve as important diagnostic and prognostic markers for various infectious and inflammatory diseases. In the present study, the effects of melatonin on expression of the positive APPs *SAA*, *haptoglobin*, *CRP*, *ceruloplasmin*, and *α-1 antitrypsin*, and of the negative APP *fibrinogen* in LPS-stimulated bMECs were investigated. Only the effect of melatonin on *CRP* has been described previously, in a study of diabetes-associated low-grade inflammation (Agil *et al.*, 2012). Results of the present study show that melatonin reduced the expression of all of the positive APPs and significantly increased the mRNA level of the negative APP.

An inflammatory response activates the synthesis of immunomodulators that result in cataclysmic levels of ROS and RNS (Reuter *et al.*, 2010). These toxicants trigger the discharge of previously sequestered Ca^{2+} into the cytosol and cause mitochondrial lesions that result in the release of cytochrome c and activation of the apoptotic cascade (Reiter *et al.*, 2016). An effective anti-inflammatory therapeutic

strategy needs to be able to reduce the production of inflammatory mediators and to abate oxidative stress initiated by inflammation (Barrett *et al.*, 2010). Melatonin is a powerful antioxidant. It 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 the cell function. In the present study, melatonin reversed the LPS-induced reduction in dityrosine levels and suppressed nitrite levels. Dityrosine is a fluorescent molecule generated during normal post-translational processes. Because tyrosine dimerization, as well as nitration, can be affected by peroxynitrite, dityrosine is considered a biomarker of oxidatively modified proteins (Hattori *et al.*, 2015). At peroxynitrite levels of 5 μM or less, tyrosine is almost exclusively dimerized to give dityrosine, 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 inflammatory conditions (Soulere *et al.*, 2001). Results of the present study indicate that melatonin reduced oxidative protein damage caused by LPS stimulation. NO is produced from L-arginine by the enzyme NO synthase. It is an important intracellular and extracellular signaling molecule involved in diverse biological processes, including regulation of vascular tone, neurotransmission, and the immune response, and inhibition of platelet aggregation (Andrukhov *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 many toxic effects,

causing nitrosative stress (Hughes, 2008). Reductions in NO levels are beneficial to cells, precluding LPS action and reducing inflammation. NO released by cultured cells into the medium is readily oxidized to nitrite (Pinho *et al.*, 2011). Nitrite levels are significantly increased under inflammatory conditions (Pinho *et al.*, 2011; Nishikawa *et al.*, 2012). The results of this study reveal that levels of nitrite in the culture medium of LPS-stimulated bMECs were markedly suppressed by pretreatment with melatonin. Therefore, melatonin prevented nitrosative stress caused by LPS stimulation. In the present study, melatonin also activated the Nrf2 antioxidant defense pathway. Nrf2 is an important sensor of oxidants. Upon activation, Nrf2 translocates to the nucleus and interacts with the antioxidant response element to initiate transcription of HO-1 (Tamaki *et al.*, 2014). HO-1 enhances cellular resistance to oxidative stress and protects against inflammation (Huang *et al.*, 2013). In the present study, melatonin protected bMECs from LPS-induced inflammatory and oxidant stress damage.

Abstract

Mastitis is the most prevalent disease in dairy cattle worldwide and not only causes huge economic losses in the dairy industry but also threatens public health. To evaluate the therapeutic potential of melatonin in mastitis, the ability of melatonin to protect bovine mammary epithelial cells (bMECs) from the harmful effects of lipopolysaccharide (LPS) was examined. The result shows that melatonin inhibited the LPS-binding protein–CD14–TLR4 signaling pathway in bMECs, which had opposing effects on pro-inflammatory and anti-inflammatory mediators. Melatonin decreased LPS-induced expression of pro-inflammatory cytokines, chemokines, and positive acute-phase proteins (APPs), including *tumor necrosis factor-α*, *interleukin* (*IL*)*-1β*, *IL-6*, *granulocyte-monocyte colony-stimulating factor*, *chemokine CC motif ligand* (*CCL*)*2*, *CCL5*, *serum amyloid A*, *haptoglobin*, *C-reactive protein*, *ceruloplasmin*, and *α-1 antitrypsin*, and increased expression of the anti-inflammatory cytokine *IL-1Ra* and the negative APP *fibrinogen*. In addition, melatonin increased dityrosine levels but suppressed nitrite levels by upregulating the expression of *Nrf2* and *heme oxygenase-1* in the Nrf2 antioxidant defense pathway. Finally, melatonin administration increased the viability of LPS-stimulated bMECs. These results suggest that melatonin protects bMECs from LPS-induced inflammatory and oxidant stress damage and provide evidence that melatonin might have therapeutic utility in mastitis.

Chapter 3

Protective effect of melatonin on LPS-stimulated granulosa cells in the Japanese quail

3.1 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 (Takata *et al.*, 2003). In an avian colibacillosis case, the *E. coli* can invade this layer, cause severe inflammation and a low laying rate (Kabir, 2010). 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 (Pieterse and Todorov, 2010; 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). Previous studies have shown the ability of melatonin to repress pro-inflammatory cytokine levels and reduce oxidative stress in experimental inflammation as mentioned in the Chapter 2. However, the anti-inflammatory effect of melatonin on granulosa cells has not been investigated. Here, the effects of melatonin on the mRNA levels of pro-inflammatory cytokines and chemokine in LPS-stimulated granulosa cells *in vitro* were investigated. Additionally, ROS/RNS levels in the cell culture medium and evaluated the potential benefits of melatonin on cell viability following LPS treatment were examined. Finally, the effect of high doses of melatonin on progesterone basal secretion by granulosa cells was investigated.

3.2 Materials and methods

3.2.1 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.

3.2.2 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 μL 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 × *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 1×10^6 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_2 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 μg/mL, according to a previous study (Xia *et al.*, 2012). Granulosa cells were pretreated with or without melatonin for 12 h and then 100 ng/mL LPS (from *E. coli*; ALX-581-014-L001; Enzo Life Sciences, Farmingdale, NY, USA) (Shao *et al.*, 2015) was added to the culture medium stimulating 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.

3.2.3 RNA isolation and cDNA preparation

Total RNA was extracted using NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. The total RNA was treated with DNase I at 18–25 °C for 15 min. The RNA was quantified by measuring the optical density at a wavelength of 260 nm using an OD260 unit equivalent to 40 μg/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).

3.2.4 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 μL SYBR Premix Ex Taq II, 0.4 μM forward and reverse primers, 0.4 μL ROX reference dye, and 2 μL template, and ddH2O was added to a total volume of 20 μL. 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 $2^{-\Delta\Delta Ct}$ method. Samples from six quails were analyzed.

3.2.5 ELISA

Cell culture medium was precleared by centrifugation at 3,000 × *g* for 20 min to remove cells and stored at −20°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 μM. Samples from six quails were analyzed in duplicate.

For the progesterone assay, cell-free culture medium was extracted in advance as described previously was prepared as described previously (Isobe *et al.*, 2005; Isobe *et al.*, 2007). The culture medium was mixed with 2 mL petroleum ether (Kanto Chemical Co., Tokyo, Japan) and shaken for 15 min. After decantation, the ether phase was evaporated in a glass tube. Borate buffer, consisting of 0.05 M boric acid

(Kanto Chemical Co.), 0.2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1 mg/mL thimerosal (Kanto Chemical Co.), was added into tubes for reconstitution, followed by an enzyme immunoassay with an anti-progesterone antibody. The 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, 5α-pregnanedione, 20β-hydroxyprogesterone, deoxycorticosterone, pregnenolone, 5β-pregnane-3α-ol-20-one, and 17α-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.

3.2.6 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.

3.2.7 MTT Assay

The viability of cultured granulosa cells was quantified using an MTT assay. Briefly, cells were seeded in 96-well plates at 1×10^5 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 μL 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.

3.2.8 Statistical Analysis

Continuous variables are expressed as the mean ± 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.

Table 3.1. Primers used for qRT-PCR.

Gene	Primer sequence (5′–3′)	Accession no.	Product size (bp)
IL-1β	Forward: GGGCATCAAGGGCTACAA	NM_204524.1	138
	Reverse: CTGTCCAGGCGGTAGAAGAT		
IL-6	Forward: AGAAATCCCTCCTCGCCAAT	NM_204628.1	121
	Reverse: AAATAGCGAACGGCCCTCA		
IL-8	Forward: GGCTTGCTAGGGGAAATGA	HM179639.1	200
	Reverse: AGCTGACTCTGACTAGGAAACTGT		
GADPH	Forward: ATCACAGCCACACAGAAGACG	M11213	124
	Reverse: TGACTTTCCCCACAGCCTTA		

3.3 Results

LPS caused substantial increases in the mRNA levels of *IL-1β*, *IL-6*, and *IL-8* (Figure 3.1). Addition of 10 μg/mL melatonin significantly decreased *IL-1β* mRNA level ($P<0.05$) (Figure 3.1A), whereas *IL-6* and *IL-8* mRNA levels were significantly decreased by melatonin at concentrations of 10 and 100 μg/mL ($P<0.05$) (Figures 3.1B, and 3.1C).

To examine oxidative stress, the dityrosine level in the supernatants of treated granulosa cells was measured. ELISA showed that LPS substantially decreased the production of dityrosine ($P<0.05$), but this effect was inhibited by pretreatment with 10 and 100 μg/mL melatonin (Figure 3.2).

To evaluate nitrative stress, the level of nitrite in the culture medium was determined by the Griess reaction; it was markedly increased by LPS stimulation ($P<0.05$) (Figure 3.3). As expected, melatonin significantly suppressed the nitrite levels at both 10 and 100 μg/mL ($P<0.05$).

The viability of cultured granulosa cells was examined using the MTT assay. Although LPS significantly reduced the cell viability ($P<0.05$), melatonin at concentrations of 10 and 100 μg/mL prevented cell death (Figure 3.4).

The basal secretion of progesterone was checked by ELISA. No significant differences were detected between any of the treatments ($P>0.05$) (Figure 3.5).

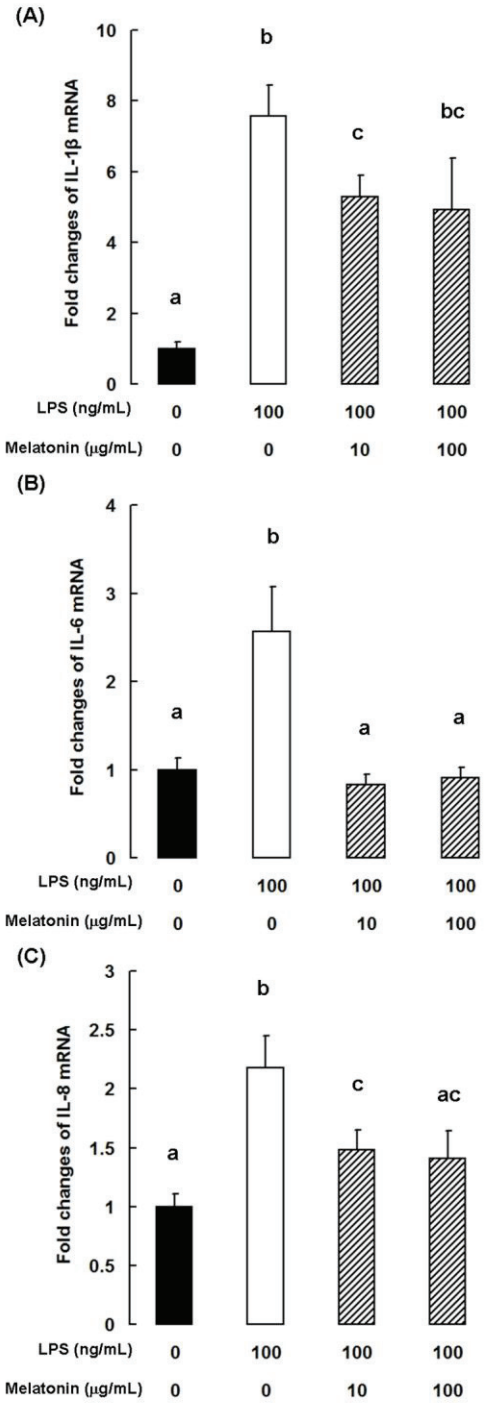

Figure 3.1. Effect of melatonin on LPS-induced cytokine and chemokine mRNA expression. Melatonin modulates LPS-induced cytokine mRNA expression. qRT-PCR analysis of (A) *IL-1β*, (B) *IL-6*, and (C) *IL-8* mRNA in cultured granulosa cells treated with the indicated combinations of LPS and melatonin. Data are the mean ± SD of six independent experiments. Values with different superscripts are significantly different at $P<0.05$.

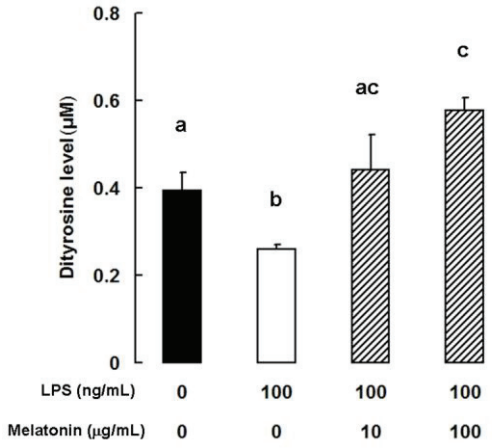

Figure 3.2. Effect of melatonin on dityrosine level in LPS-stimulated granulosa cells. Cells were treated with the indicated combinations of LPS and melatonin, and culture supernatants were analyzed for the presence of dityrosine by ELISA. Data are the mean ± SD of six independent experiments, each measured in duplicate. Values with different superscripts are significantly different at $P<0.05$.

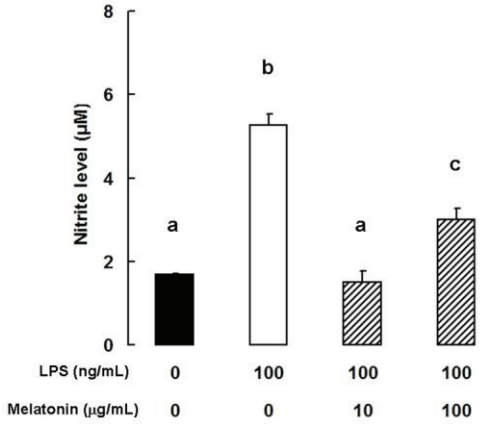

Figure 3.3. Effect of melatonin on nitrite level in LPS-stimulated granulosa cells. Cells were treated with the indicated combinations of LPS and melatonin, and culture supernatants were analyzed for the presence of nitrites by the Griess reaction. Data are the mean ± SD of six independent experiments, each measured in duplicate. Values with different superscripts are significantly different at $P<0.05$.

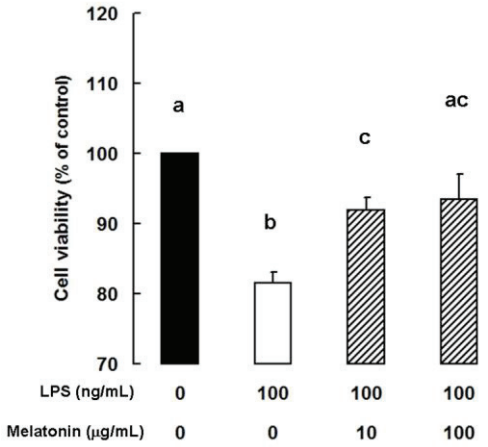

Figure 3.4. Effect of melatonin on LPS-induced cell death. Cell viability was quantified using the MTT assay. Data are the mean ± SD of three independent experiments, each measured in sextuplicate. Values with different superscripts are significantly different at $P<0.05$.

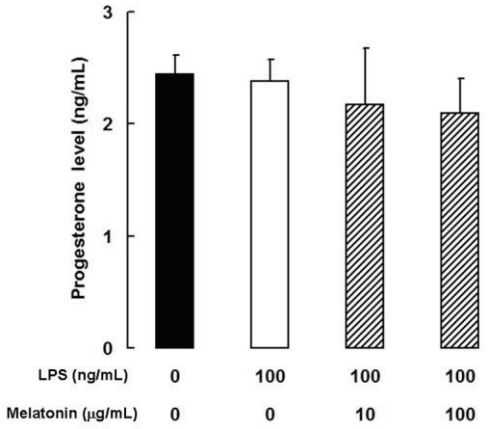

Figure 3.5. Progesterone secretion by cultured granulosa cells. The level of progesterone was measured by ELISA. Data are the mean ± SD of six independent experiments, each measured in duplicate.

3.4 Discussion

Cytokines are a broad and loose category of small, nonstructural, secreted proteins that are synthesized by and induce a response in nearly all nucleated cells (Dinarello, 2010). 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-1β*, *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-1β*, *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 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 μM 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 (Soulere *et al.*, 2001). Thus, the inhibition of the LPS-induced decrease in dityrosine levels by melatonin pretreatment in this 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 (Andrukhov *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; Nishikawa *et al.*, 2012). Results of the presnt study show that level of nitrite in the culture medium of LPS-stimulated granulosa cells was significantly suppressed by pretreatment with melatonin. Therefore, melatonin prevented nitrosative stress caused by LPS stimulation *in vitro*. The findings are consistent with a previous observation that modulation of apoptosis requires high melatonin doses (Radogna *et al.*, 2007; Radogna *et al.*, 2009). Xia *et al.* (2012) showed that melatonin at 10–232 μg/mL effectively inhibited LPS-induced inflammation. Both concentrations of 10 and 100 μg/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. Results of this study 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.

Abstract

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 the Japanese quail. Granulosa cells isolated from the Japanese quails were pretreated with or without melatonin (10 or 100 μg/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. Results show that melatonin decreased LPS-induced expression of *IL-1β*, *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, such as avian colibacillosis in the Japanese quail.

Chapter 4

Melatonin does not affect progesterone basal secretion but suppresses the luteinizing hormone receptor expression in granulosa cells of the Japanese quail

4.1 Introduction

The multi-function of melatonin in both plants and animals mentioned in the Chapter 1 indicates that melatonin is a more universal molecule than previously thought, with functions in various tissues and organs. In mammals, melatonin acts on the ovaries, with direct effects on granulosa cell steroidogenesis and follicular functions (Wang *et al.*, 2012). Melatonin directly stimulates progesterone production by human and bovine granulosa cells *in vitro* (Webley and Luck, 1986). Perfusion with melatonin markedly stimulated progesterone secretion by the marmoset corpus luteum (Webley and Hearn, 1987). Melatonin also improves the concentration of plasma progesterone in sheep and rats (Abecia *et al.*, 2002; Chuffa *et al.*, 2013). However, there are some notable differences between birds and mammals with regard to ovarian function and divergent characteristics of the biosynthesis and role for progesterone. Poultry birds maintain a strict follicular hierarchy consisting of approximately 2 to 6 pre-ovulatory follicles, and ovulate a single follicle almost every day (McDerment *et al.*, 2012). In nearly all species of birds, the synthesis of progesterone within the follicular granulosa is a requirement for ovulation,

comparable to the site of synthesis and role of estradiol in mammals (Johnson, 2014). Murayama *et al.* (1997) indicated the direct action of melatonin on hen ovarian granulosa cells to lower their responsiveness to luteinizing hormone (LH) in vitro. However, reports on the effects of melatonin on progesterone production under basal conditions and the expression of the LH receptor (LHCGR) in birds are not available.

In the present study, the effect of melatonin on progesterone production by granulosa cells *in vitro* at a wide range of concentrations in F1–F3 follicles without LH stimulation was investigated. The expression of melatonin receptor subtypes and the level of *LHCGR* mRNA in cultured granulosa cells from F1 follicles stimulated by melatonin were also examined. Finally, the effect of melatonin on progesterone production *in vivo* was investigated.

4.2 Materials and methods

4.2.1 Experimental birds

In total, 36 female Japanese quails, 15–30 weeks of age, were used. 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*. For the *in vitro* experiment, birds were decapitated to collect pre-ovulatory follicles when the F1 follicles were predicted to be immature or mature, 3–6 or 18–21 h after oviposition, respectively (Reece, 2004). F1–F3 follicles were collected at the same time. For the *in vivo* experiment, animals were intraperitoneally injected with melatonin (0.67 mg/kg body weight; a concentration similar to that used for rat injection [Abd-Allah *et al.*, 2003; Chuffa *et*

al., 2013]) or vehicle at 3 and 18 h after oviposition. The serum level of melatonin after administration was estimated as 8.38 μg/mL, based on the method of Ito *et al.* (2011). The birds were decapitated to collect serum 3 h later (at 6 and 21 h after oviposition). All animals used in this study were handled in accordance with the regulations of the Animal Experiment Committee of Hiroshima University for animal experiments.

4.2.2 Cell culture and treatment

Granulosa cells were isolated as previously described (Rangel *et al.*, 2009). In brief, pre-ovulatory F1–F3 follicles were placed in cell-culture dishes containing preheated 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 sterile DPBS. The granulosa layers detached from the hierarchical follicles were disaggregated at 37°C for 5 min under continuous agitation in 500, 300, and 200 μL of dissociation solution (CTK; ReproCELL, Yokohama, Japan). The enzymatic reaction was quenched by the addition of 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco BRL/Invitrogen, Karlsruhe, Germany) containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Cells pellets were flushed with a pipette, and centrifuged. Cells were washed three times by resuspension in 5 mL DPBS and centrifuged for 5 min at 500 × *g*. Granulosa cells were seeded at a concentration of 2×10^5 cells/mL in DMEM/F12 medium containing 10% (v/v) FBS,

1% (v/v) nucleosides (Millipore, Billerica, MA, USA), 1% (v/v) non-essential amino acids (Gibco BRL/Invitrogen), 1 mM sodium pyruvate (Gibco BRL/Invitrogen), and 1% (v/v) antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan). Cells were cultured at 39°C in a humidified atmosphere with 5% CO_2 and 95% air.

A stock solution of melatonin was prepared by dissolving 10 mg of melatonin (Sigma-Aldrich) in 200 μL of ethanol. Working solutions were prepared by dilution in cell culture medium to give a concentration gradient of 0.0001–100 μg/mL. Granulosa cells were treated with different concentrations of melatonin for 12 h, a culture time used previously (Taketani *et al.*, 2011). As controls, cells were incubated with ethanol at the highest concentration used for melatonin treatment.

4.2.3 RNA extraction

At least 5×10^5 cultured granulosa cells from F1 follicles were used for total RNA extraction. The cell culture medium was aspirated. Adherent cells were washed once with 2 mL ice-cold DPBS, which was then aspirated as much as possible. The cells were used for total RNA extraction with the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instruction. The obtained RNA pellet was treated with DNase I (Macherey-Nagel) and dissolved in diethyl pyrocarbonate-treated water (Nacalai Tesque). The total RNA was quantified by measuring the optical density at a wavelength of 260 nm using an OD260 unit equivalent to 40 μg/mL of RNA. The RNA purity was determined by measuring the ratio of absorbance at 260 and 280 nm. Only samples with a ratio between 1.8 and 2.2

were used.

4.2.4 Reverse transcription (RT)-PCR

The RNA solution was incubated at 65°C for 5 min and kept on ice afterwards. The total RNA was reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) to obtain the cDNA. The reaction mixture consisted of 240 ng of total RNA, 5× reverse transcription buffer, 0.5 μL reverse transcription enzyme mixture, and 0.5 μL primer mixture, and nuclease-free water was added to a total volume of 10 μL. Reverse transcription was performed at 37°C for 15 min, followed by heat inactivation for 5 min at 98°C using an Applied Biosystems GeneAmp PCR System 9700 (Life Technologies, Darmstadt, Germany).

The RT-PCR mixture consisted of 0.25 μL TaKaRa Ex Taq, 5 μL 10× Ex Taq buffer, 4 μL dNTP mixture, 0.5 μM each of forward and reverse primers, 1 μL template, and double distilled water to a total volume of 20 μL. The reaction procedure was as follows: initial denaturation at 98°C for 2 min, followed by 50 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. A final extension step was performed at 72°C for 7 min. cDNA products were resolved on 2% (w/v) agarose gels containing 0.025% (w/v) ethidium bromide. The analysis was repeated thrice.

4.2.5 Analysis of the difference in LHCGR expression

The *LHCGR* mRNA levels in cultured granulosa cells of F1 follicles was

analyzed using quantitative real-time PCR and the SYBR Premix Ex Taq II (Takara Bio., Shiga, Japan) on an Applied Biosystems StepOne real-time PCR system (Life Technologies) according to the method described previously (Guangmin *et al.*, 2015). In brief, the PCR mixture (20 μL) consisting of 10 μL SYBR Premix Ex Taq II, 0.4 μM each of forward and reverse primers, 0.4 μL ROX reference dye, 2 μL template, and 6 μL double distilled water were mixed in PCR tubes (Life Technologies). The thermal protocols for PCR were as follows: initial denaturation at 95°C for 30 s followed by 50 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 34 s, and a melting curve from 60 to 95° C, increasing in increments of 0.5°C every 5 s. Normalization was performed using the GAPDH housekeeping gene as a control. Primer sequences are listed in Table 4.1. Real-time PCR data were analyzed using the $2^{-\Delta\Delta ct}$ method.

4.2.6 Measurement of progesterone levels

Cell culture medium or blood sample was pre-cleared by centrifugation at 3,000 × *g* for 20 min to remove cells and then stored at −20°C until further use. The progesterone assay was performed as described in the Chapter 3.

4.2.7 Statistical analysis

Continuous variables are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) or two-way ANOVA followed by Duncan's

multiple-range test with the Statview software (Abacus Concepts, Berkeley, CA, USA). Differences were considered statistically significant when the *P* value was less than 0.05.

Table 4.1. Primers used for PCR.

Gene	Primer sequence (5′–3′)	Accession no.	Product size (bp)
Mel-1a	Forward: CAATGGATGGAATCTGGGA	NM_205362.1	333
	Reverse: GCTATGGGAAGTATGAAGTGG		
Mel-1b	Forward: TTTGCTGGGCACCTCTAAAC	NM_001293103.1	259
	Reverse: CGCTTGCTCTTCTGTCCATC		
Mel-1c	Forward: AGATAAGTGGGTTCCTGATGG	NM_205361.1	237
	Reverse: GCAAAGGTGCAAGAGTAAATC		
LHCGR	Forward: TTGCACATTGAGGACGGAGC	NM_204936.1	194
	Reverse: GATTCGTTGCTCATGCCCTG		
GADPH	Forward: ATCACAGCCACACAGAAGACG	M11213	124
	Reverse: TGACTTTCCCCACAGCCTTA		

Mel-1a, melatonin receptor 1a; *LHCGR*, luteinizing hormone receptor.

4.3 Results

Figure 4.1 shows the effect of melatonin, follicle size, and collection time post-oviposition on the secretion of progesterone by pre-ovulatory granulosa cells cultured for 12 h. No statistical differences were observed between progesterone secretion by granulosa cells with or without melatonin administration ($P>0.05$). When F1 follicle granulosa cells were cultured with 0.1 and 1 μg/mL melatonin, progesterone secretion by cells collected at 3–6 h after oviposition was significantly less than by cells collected at 18–21 h after oviposition ($P<0.05$), but no differences between the two collection times were observed at other melatonin concentrations (Figure 4.1A). Progesterone secretion by the granulosa cells from F2 follicles was remarkably less for cells collected at 3–6 h than collected at 18–21 h after oviposition when they were cultured with 0, 0.0001 and 1μg/mL melatonin ($P<0.05$), but this difference disappeared in the presence of other concentrations of melatonin (Figure 4.1B). The secretion of progesterone by F3 granulosa cells was significantly lower for cells collected at 3–6 h after oviposition than at 18–21 h after oviposition at nearly all concentrations, except 0.0001 and 0.1 μg/mL melatonin (Figure 4.1C).

The expression profile of melatonin receptor subtypes in cultured granulosa cells from F1 follicles revealed that melatonin receptor subtypes (*Mel-1a*, *1b* and *1c*) were expressed in granulosa cells of the Japanese quail (Figure 4.2).

The effect of melatonin on the expression of *LHCGR* mRNA in cultured granulosa cells from F1 follicles was shown in Figure 4.3. Melatonin significantly suppressed *LHCGR* mRNA expression after culturing for 12 h at two doses used

(0.0001 and 0.001 μg/mL) (P<0.05) in cells collected at both 3–6 h and 18–21 h after oviposition.

With regard to the *in vivo* experiment, no statistical differences were observed between progesterone levels in serum of quails at 6 h after oviposition with or without melatonin administration (P>0.05). However, for animals injected with melatonin, the serous progesterone level was significantly less at 21 h after oviposition (P<0.05) (Figure 4.4).

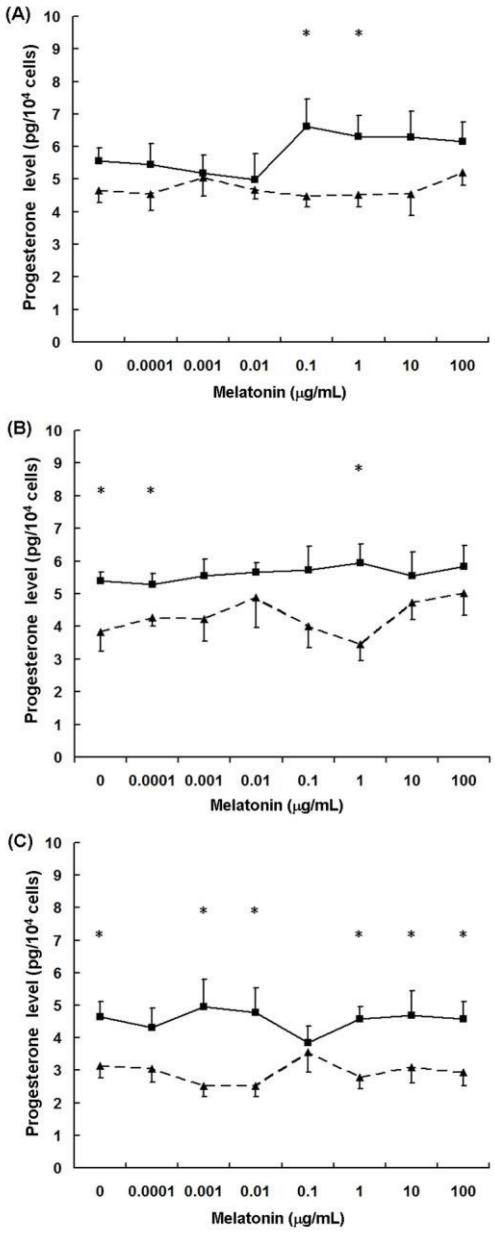

Figure 4.1. Progesterone secretion by granulosa cells of Japanese quail cultured for 12 h with or without melatonin stimulation. Granulosa cells were obtained from (A) F1, (B) F2, or (C) F3 follicles, at 3–6 h (triangles, dashed line) or 18–21 h (squares, solid line) after oviposition. Data are the mean ± SD (n=6). *Values are significantly different between 3–6 h group and 18–21 h group ($P<0.05$).

Figure 4.2. The expression of melatonin receptor subtypes in cultured granulosa cells from F1 follicles of the Japanese quail. The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. M = marker.

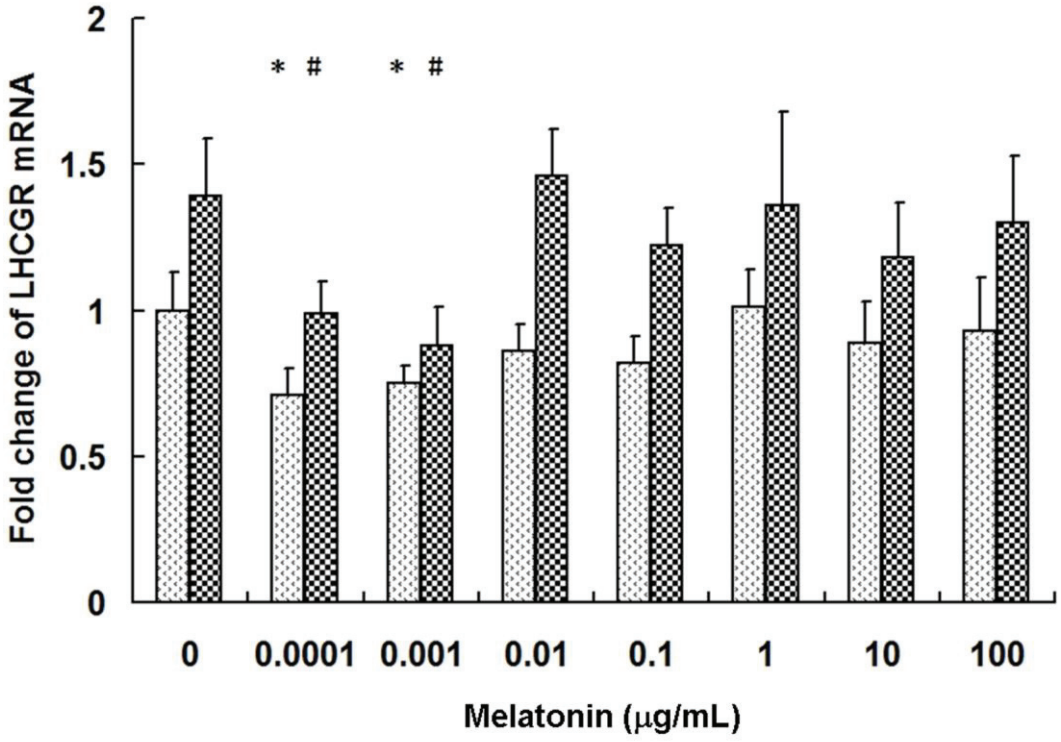

Figure 4.3. The effect of melatonin on the expression of *LHCGR* mRNA in cultured granulosa cells from F1 follicles of the Japanese quail. Histograms filled with arrowheads present data of 3–6 h, while filled with squares present data of 18–21 h. Data are the mean ± SD (n=6). *,#Values are significantly different compared to the values of 0 g/mL ($P<0.05$).

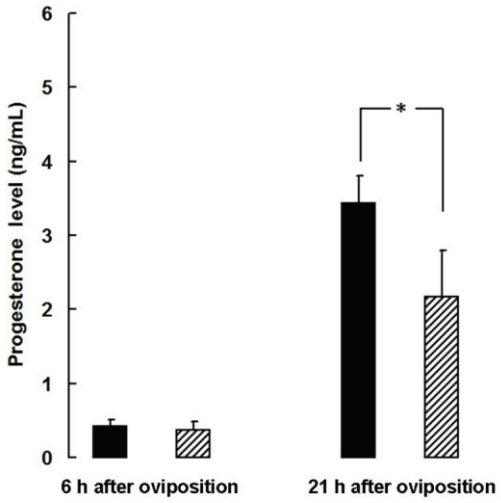

Figure 4.4. Progesterone level in serum of the Japanese quail with or without melatonin administration. Black histograms present data of the controls, while histograms filled with oblique lines present data of melatonin treatment. Data are the mean ± SD (n=6). *Values are significantly different at $P<0.05$.

4.4 Discussion

Progesterone plays a key role in regulating the reproductive activity (Yu and Maeda, 2017). In birds, ovarian follicles maintain a monolayer of granulosa cells (Diez-Fraile *et al.*, 2010). The pre-ovulatory follicles grow in size due to yolk incorporation and the granulosa cells produce progressively greater amounts of progesterone (Onagbesan *et al.*, 2009; Sechman, 2013; Johnson, 2014). Granulosa cells can be collected from pre-ovulatory follicles of poultry bird and cultured *in vitro*, which provides a means for the study of progesterone biosynthesis in avian species. In the present study, the effect of melatonin on progesterone secretion by granulosa cells collected from F1–F3 pre-ovulatory follicles at 3-6 h and 18-21 h after oviposition without LH stimulation *in vitro* was investigated. The result showed that melatonin does not affect the basal secretion of progesterone in cultured granulosa cells of the Japanese quail.

The pre-ovulatory progesterone surge is predominantly derived from the granulosa layer of the largest mature pre-ovulatory follicle (F1) (Etches, 1994). The pre-ovulatory F1 follicle produces 30 times more progesterone than the F2 and F3 follicles in the absence of exogenous LH *in vitro* (Yu *et al.*, 1992) and granulosa cells of the F1 follicle are more responsive to LH stimulation than granulosa cells of the F3 follicle (Robinson *et al.*, 1988). The pre-ovulatory release of LH is stimulated by the positive feedback action of progesterone in avian species (Johnson *et al.*, 1985; Johnson *et al.*, 2002). In addition, adequate amounts of granulosa cells can be isolated from F1 follicles of the Japanese quail. Thus, the expression of melatonin receptor

subtypes and the effect of melatonin on *LHCGR* mRNA in granulosa cells of the F1 follicles were investigated in the present study. Result show that melatonin significantly suppressed *LHCGR* mRNA expression in the relatively lower concentrations used in this study. Moreover, decreased *LHCGR* expression suggests a concordant decrease in the responsiveness of melatonin-treated granulosa cells to LH.

To substantiate the above assumption, the effect of melatonin on progesterone production *in vivo* was investigated. There were no statistical differences between serum progesterone levels of Japanese quails at 6 h after oviposition with or without melatonin administration, which is similar to the basic conditions observed *in vitro*. The release of LH from the pituitary relates to the degree of maturation of the pre-ovulatory follicles (Etches, 1994). LH is not released from the pituitary when the F1 follicles are predicted to be immature (at 3-6 h after oviposition), maintaining LH at low basic levels in circulating blood (Hrabia *et al.*, 2014). At 18-21 h after oviposition, the F1 follicles are predicted to mature, and the pituitary starts releasing LH into circulating blood (Reece, 2004). The progesterone level in blood reaches a peak with a surge in LH levels (Nakagawa-Mizuyachi *et al.*, 2010). In the present study, at 21 h after oviposition, the serous progesterone level was significantly lower in quails injected with melatonin, which is in agreement with an earlier *in vitro* study performed by Murayama *et al.* (1997). In their study, the dose-response curve for LH-stimulated progesterone production of hen granulosa cells shifted to a higher concentration of LH to attain ED50 in the presence of melatonin. Therefore, these effects of melatonin on progesterone production are consistent with the decreased

LHCGR mRNA expression demonstrated by the present study. The findings in this study and published information can explain, at least in part, the phenomenon of longer nocturnal hours being de-stimulatory in poultry bird reproductive cycle. Prolonged nocturnal hours stimulate melatonin biosynthesis in the pineal gland, which subsequently increases melatonin release in the bloodstream. The elevated concentration of melatonin in circulating blood inhibits *LHCGR* mRNA expression in the ovarian granulosa cells, which thereby reduces the LH responsiveness of granulosa cells in the pre-ovulatory follicles. With the decrease in LH-stimulated progesterone production by granulosa cells, the serum progesterone level decreases, which weakens the positive feedback action of progesterone on the pre-ovulatory release of LH. Thus, decreased serum LH levels ulteriorly reduce progesterone production by granulosa cells. These findings can also explain why short-day photoperiod or administration of melatonin decreases the ovary weight of developing avian species (Darre, 2011). Accumulating evidence may account for the fact that poultry birds produce well on long days and short nights.

Abstract

The aim of this study was to evaluate the potential effect of melatonin on progesterone production by granulosa cells of the Japanese quail. For *in vitro* experiments, granulosa cells were isolated from pre-ovulatory follicles (F1–F3) when the F1 follicles were predicted to be either immature or mature (at 3-6 or 18-21 h after oviposition, respectively). Granulosa cells were cultured for 12 h with or without melatonin concentration gradients of 0.0001–100 μg/mL, thereby averting luteinizing hormone (LH) stimulation. The concentration of progesterone in culture medium was measured using an enzyme immunoassay. The expression of melatonin receptor subtypes in granulosa cells from F1 follicles was detected by reverse transcription-PCR. The *LH receptor* (*LHCGR*) mRNA level in cultured granulosa cells of the F1 follicles was analyzed using quantitative real-time PCR. Six quails were used in each of four groups for *in vivo* experiments. Each group received intraperitoneal injection of melatonin (0.67 mg/kg body weight) or mock-vehicle at 3 or 18 h after oviposition, respectively. The birds were decapitated to collect serum 3 h later (at 6 or 21 h after oviposition, respectively). The serum progesterone level was also measured using an enzyme immunoassay. Results showed that melatonin receptor subtypes (*Mel-1a*, *1b*, and *1c*) were expressed in the granulosa cells of the F1 follicles of the Japanese quail. Melatonin suppresses the *LHCGR* mRNA expression in granulosa cells of F1 follicles but does not affect the basal secretion of progesterone in cultured granulosa cells of the F1–F3 follicles. In addition, melatonin treatment has

no influence on the serous progesterone concentration at 6 h post-oviposition, but suppresses progesterone level 21 h after oviposition in the Japanese quail.

Chapter 5

General discussion

Provision of appropriate pharmaceutical, such as melatonin can have anti-inflammatory effects (Carrillo-Vico *et al.*, 2013). There have been only two investigations that have addressed the role of melatonin on mastitis model. Boulanger *et al.* (2002) evaluated the effect of melatonin in bovine neutrophil-induced mammary cell damage. The protective effect of melatonin on a mouse mastitis model was reported recently (Shao *et al.*, 2015). However, it was uncertain that the effect of melatonin on LPS-induced bovine mammary epithelial cell (bMEC) inflammation. The observations obtained in the Chapter 2 indicate that melatonin inhibited the LPS-binding protein–CD14–TLR4 signaling pathway in bMECs, which had opposing effects on pro-inflammatory and anti-inflammatory mediators. Melatonin decreased LPS-induced expression of pro-inflammatory cytokines, chemokines, and positive acute-phase proteins (APPs), including tumor necrosis factor-α, interleukin (IL)-1β, IL-6, granulocyte-monocyte colony-stimulating factor, chemokine CC motif ligand (CCL)2, CCL5, serum amyloid A, haptoglobin, C-reactive protein, ceruloplasmin, and α-1 antitrypsin, and increased expression of the anti-inflammatory cytokine IL-1Ra and the negative APP fibrinogen. In addition, melatonin increased dityrosine levels but suppressed nitrite levels by upregulating the expression of Nrf2 and heme

oxygenase-1 in the Nrf2 antioxidant defense pathway. Finally, melatonin administration increased the viability of LPS-stimulated bMECs. The results confirm the hypothesis that melatonin can protect the bMECs from the LPS-induced cell damage. It is hopeful to administer melatonin to dairy cows with clinical and subclinical mastitis in the field in future.

In the Chapter 3, beneficial effects were observed when melatonin was administered to LPS-stimulated cultured granulosa cells of the Japanese quail. Melatonin decreased LPS-induced expression of *IL-1β*, *IL-6*, *IL-8*, and suppressed the nitrite level. On the contrary, melatonin increased the dityrosine level. In addition, melatonin administration increased the viability of LPS-stimulated granulosa cells *in vitro*. 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, such as avian colibacillosis in the Japanese quail.

However, it is reported that there are also several effects of melatonin on animal reproduction physiological processes (Wang *et al.*, 2014). In mammals, melatonin acts on the ovaries, with direct promotion effects on granulosa cell steroidogenesis and follicular functions (Webley and Luck, 1986; Webley and Hearn, 1987; Abecia *et al.*, 2002; Chuffa *et al.*, 2013). With regard to ovarian function and divergent characteristics of the biosynthesis and role for progesterone, there are notable differences between birds and mammals. In order to test this postulation, whether exposure of granulosa cells to melatonin would create changes in progesterone

production was determined in the Chapter 4. It was found that melatonin receptor subtypes (*Mel-1a*, *1b*, and *1c*) were expressed in the granulosa cells of the pre-ovulatory F1 follicles of the Japanese quail. Melatonin suppresses the *LHCGR* mRNA expression at low concentrations in granulosa cells of F1 follicles but does not affect the basal secretion of progesterone in cultured granulosa cells of the F1–F3 follicles. In addition, melatonin treatment at a low concentration has no influence on the serum progesterone concentration at 6 h post-oviposition, but suppresses progesterone level 21 h after oviposition in the Japanese quail. These results demonstrated that only the low melatonin concentrations had negative effects on progesterone production of the Japanese quail; with the drastically exceeding physiological melatonin used in the anti-inflammatory experiment, no harmful effects were detected under the present situation.

In this work, it has been found that 1) melatonin can protect the bMECs and granulosa cells of the Japanese quail from the LPS-induced cell damage; 2) there are no harmful effects of the drastically exceeding physiological melatonin on progesterone production of the Japanese quail. Based on these findings, it is hopeful to administer melatonin to dairy cows with clinical and subclinical mastitis and quails with avian colibacillosis in the field to supersede the overuse of antibiotics in the future, and therefore may contribute to the improvement of livestock industry.

Chapter 6

General summary

The overuse of antibiotics threatens both the development of livestock industry and the public health. The use of antibiotics in animal food production will become even more severely restricted in the future. Provision of appropriate pharmaceutical, such as melatonin can have some anti-inflammatory effects in the experimental animals. The goal of this study was in an attempt to test the anti-inflammatory effects of melatonin on LPS-stimulated bovine mammary epithelial cells (bMECs) and granulosa cells of the Japanese quail, and also to evaluate its potential effects on progesterone production of the Japanese quail.

The effect of melatonin on LPS-induced bMEC inflammation was examined in the Chapter 2. Melatonin treatment of bMECs attenuated the LPS-stimulated increase in pro-inflammatory cytokine, chemokine, and positive APP mRNA but increased the expression of an anti-inflammatory cytokine and a negative APP by inhibiting the TLR4 signaling pathway. In addition, melatonin inhibited oxidative stress by activating the Nrf2 antioxidant defense pathway. Finally, melatonin treatment had a beneficial effect on the viability of LPS-stimulated bMECs.

In the Chapter 3, the anti-inflammatory effect of melatonin on cultured granulosa cells of the Japanese quail was evaluated. Melatonin treatment of cultured granulosa

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

There are several effects of melatonin on reproduction physiological processes in mammals. Whether the drastically exceeding physiological melatonin used in the anti-inflammatory experiment would create changes in progesterone production was determined in the Chapter 4. Melatonin at low concentrations suppresses progesterone secretion in mature pre-ovulatory follicles by down-regulating the *LHCGR* mRNA expression in the Japanese quail, with no harmful effects at the high levels used in the anti-inflammatory experiment.

In conclusion, melatonin can protect the bMECs and granulosa cells of the Japanese quail from the LPS-induced cell damage. It should contribute to the improvement of livestock industry and the public health.

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