RESEARCH ARTICLE

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Evidence of Melatonin Synthesis in the Cumulus Oocyte Complexes and its Role in Enhancing Oocyte Maturation In Vitro in Cattle

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SUMMARY

Melatonin is a multifunctional molecule that mediates several circadian and seasonal reproductive processes. The exact role of melatonin in modulating reproduction, however, is not fully understood—especially its effects on the ovarian follicles and oocytes. This study was conducted to investigate the expressions of the *ASMT* and melatonin-receptor *MTNR1A* and *MTNR1B* genes in bovine oocytes and their cumulus cells, as well as the effects of melatonin on oocyte nuclear and cytoplasmic maturation in vitro. Cumulus-oocyte complexes (COCs) from abattoir ovaries were cultured in TCM-199 supplemented with melatonin at concentrations of 0, 10, 50, and 100 ng/ml. The expression of *ASMT*, *MTNR1A*, and *MTNR1B* genes was evaluated by RT-PCR. Moreover, the effects of melatonin on cumulus cell expansion, nuclear maturation, mitochondrial characteristics and COCs steroidogenesis were investigated. Furthermore, the level of reactive oxygen species (ROS) was evaluated in denuded oocytes. Our study revealed that *ASMT* and *MTNR1A* genes were expressed in COCs, while the *MTNR1B* gene was expressed only in oocytes. Additionally, melatonin supplementation at 10 and 50 ng/ml to in vitro maturation medium significantly enhanced oocyte nuclear maturation, cumulus cell expansion and altered the mitochondrial distribution patterns, but had no effects on oocyte mitochondrial activity and COCs steroidogenesis. Melatonin-treated oocytes had a significantly lower level of ROS than controls. The presence of melatonin receptors in COCs and its promoting effects on oocyte nuclear and cytoplasmic events, indicate the potentially important roles of this hormone in regulating bovine oocyte maturation. Moreover, the presence of *ASMT* transcript in COCs suggests the possible involvement of these cells in melatonin biosynthesis.

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INTRODUCTION

In vitro handling and culture conditions expose oocytes and embryos to oxidative stressors resulting from exposure to light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates (Agarwal et al.,

Additional supporting information may be found in the online version of this article.

Abbreviations: *ASMT*, acetylserotonin O-methyltransferase; COCs, cumulus-oocyte complexes; IVM, in vitro maturation; *Mel1a*, melatonin receptor1 (also MT1); *Mel1b*, melatonin receptor2 (also MT2); MI or MII, Metaphase-I or -II; *MTNR1A*; melatonin receptor1A; *MTNR1B*, melatonin receptor 1B; ROS, reactive oxygen species.

2006) inducing detrimental chemical reactions (Mermillod et al., 2006; Tamura et al., 2008). During in vitro maturation (IVM) procedures, the environment of cumulus-oocyte complexes (COCs) provides some anti-oxidative potential to the IVM medium (Ali et al., 2003). COCs are reported to be able to produce their own protection system by secreting enzyme scavengers of reactive oxygen species (ROS) like superoxide dismutase and catalase (Cetica et al., 2001) or intracellular scavenger molecules like glutathione (de Matos and Furnus, 2000). Stimulating these endogenous protection systems through the addition of anti-oxidative molecules like mercaptoethanol, cystein, vitamins A, C, E or divalent cations chelators such as EDTA, taurine, hypotaurine, and transferrin may preserve cellular viability (Nishikimi and Yagi, 1996; Sikka, 2004; Schneider, 2005; Rodrigo et al., 2007). Supplementation of maturation medium directly with ROS scavengers such as superoxide dismutase or catalase (Blondin et al., 1997; Ikeda et al., 2005; Kobayashi et al., 2006) helped oocytes to survive challenging in vitro conditions (Mermillod et al., 2006).

Recently, attention has been directed toward melatonin as a potent and relatively cheap anti-oxidant. Melatonin and its metabolites are considered indirect antioxidants and powerful direct free radical scavengers (Adriaens et al., 2006; Kang et al., 2009a). The importance of this indole amine has been demonstrated by the presence of its receptors in reproductive organs (Woo et al., 2001). Furthermore, melatonin binding sites were identified not only in granulosa cells from human preovulatory follicles (Yie et al., 1995; Niles et al., 1999), but also in porcine cumulus and granulosa cells (Kang et al., 2009a). Two distinct receptor subtypes *Mel1a* and *Mel1b* genes (later renamed as *MT1* and *MT2* genes, respectively; Dubocovich et al., 2000; Hunt et al., 2001) have been cloned and mapped in several animal species (Reppert et al., 1994, 1995; Messer et al., 1997; Dubocovich et al., 1998; Masana and Dubocovich, 2001; von Gall et al., 2002; Alexander et al., 2007; Pandi-Perumal et al., 2008; Carcangiu et al., 2009). In mammals, the *MTNR1A (MT1)* gene seemed to be involved more in the regulation of reproductive activity than the *MTNR1B (MT2)* gene (Weaver et al., 1996; Dubocovich et al., 1998). Melatonin was reported to affect the function of testes and ovaries (Sirotkin and Schaeffer, 1997), and to alter the morphology and steroidogenesis capacity of corpus luteum, theca, and granulosa cells in vitro (Fiske et al., 1984; Baratta and Tamanini, 1992; Murayama et al., 1997; Soares et al., 2003). Furthermore, melatonin was detected in follicular fluid in humans (Brzezinski et al., 1987; Ronnberg et al., 1990). The melatonin concentrations in human follicular fluid obtained from the antral Graafian follicles were found to be significantly higher than those in collected plasma samples (Brzezinski et al., 1987; Nakamura et al., 2003). As in some other body fluids, melatonin in the follicular fluid might be concentrated against a gradient or it might be synthesized at a yet-unknown site in the ovary (possibly the granulosa cells) and released into the follicular fluid (Itoh et al., 1999). Although a recent study has revealed the importance of acetylserotonin *O*-methyltransferase (*ASMT*) in melatonin biosynthesis (Reiter et al., 2007), the presence of this enzyme has not yet been confirmed in bovine COCs.

Considering the above, it can be suggested that melatonin might have specific effects upon bovine oocyte maturation either as an antioxidant or through its receptor mechanisms. The specific objectives of this study were (i) to investigate the expression of *ASMT*, *MTNR1A*, and *MTNR1B* genes in bovine oocytes and cumulus cells; (ii) to determine the optimal melatonin concentration that can be supplemented to IVM medium of bovine COCs and assess its effects on their nuclear maturation, cumulus cell expansion, and mitochondrial activity and distribution; (iii) to study the effect of melatonin supplementation on cumulus-oocyte complex steroidogenesis; and (iv) to assess the potency of melatonin in combating ROSs in the IVM medium of bovine oocyte.

RESULTS

Melatonin Receptors in Bovine Oocytes and Cumulus Cells

The expression of the *MTNR1A* receptor gene was confirmed by using RT-PCR in both oocytes and cumulus cells before and after IVM, irrespective of melatonin addition to the IVM medium (Fig. 1, *MTNR1A*). Melatonin receptor *MTNR1B* was expressed in oocyte samples (lanes 6–10), but not in cumulus cell samples (Fig. 1, lanes 1–5, *MTNR1B*). *ASMT* gene was expressed in both cumulus cells and oocytes before and after IVM, as well as in

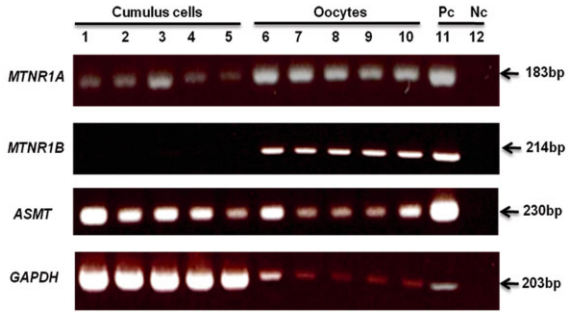

Figure 1. The expression of melatonin receptors in bovine cumulus cells and oocytes before and after IVM. RT-PCR analysis of mRNA expression of melatonin receptors *MTNR1A*, *MTNR1B*, melatonin synthesis regulator *ASMT* and the housekeeping gene *GAPDH* in the bovine cumulus cells (lines 1–5) and oocytes (lines 6–10) before and after IVM, with or without melatonin. Lanes 1 and 2 denote cumulus cell samples without melatonin treatment, before and after IVM, respectively. Lanes 3, 4, and 5 denote cumulus cell samples after IVM with 10, 50 and 100 ng/ml of melatonin, respectively. Lanes 6 and 7 denote oocyte samples without melatonin treatment, before and after IVM, respectively. Lanes 8, 9, and 10 show gene expression in oocytes after IVM with 10, 50, and 100 ng/ml of melatonin, respectively. Lanes 11 and 12 denote positive (Pc) and negative control (Nc), respectively. The housekeeping gene *GAPDH* was used as an internal control.

TABLE 1. The Effect of Melatonin Supplementation on Bovine Oocytes Nuclear Maturation In Vitro*

Melatonin (ng/ml)	No. of oocytes examined	No (% ± SEM)** of oocytes at stage				
		Dia	MI	AI	TI	MII
0	114	4 (3.5 ± 2.0)	51 (44.7 ± 2.8)[a]	3 (2.6 ± 1.4)	0 (0)	56 (49.1 ± 3.8)[b]
10	127	2 (1.5 ± 0.9)	29 (22.8 ± 3.0)[b]	3 (2.3 ± 0.7)	0 (0)	93 (73.2 ± 2.2)[a]
50	110	1 (0.9 ± 0.9)	32 (29.0 ± 4.2)[b]	2 (1.8 ± 1.8)	1 (0.9 ± 0.9)	74 (67.2 ± 4.5)[a]
100	123	6 (4.8 ± 1.8)	55 (44.7 ± 5.0)[a]	0 (0)	0 (0)	63 (51.2 ± 3.7)[b]

*Five replications of the experiment were conducted.
**Percentages are based on the number of oocytes examined.
[a,b]Values with different letters in the same column are significantly different ($P < 0.05$).
Abbreviations: Dia = diakinesis; MI = metaphase-I; AI = anaphase-I; TI = telophase-I; MII = metaphase-II.

samples treated with different doses of melatonin (Fig. 1, *ASMT*).

The Effect of Melatonin Supplementation During IVM on the Nuclear Maturation of Oocytes

The effect of different doses of melatonin on the degree of nuclear maturation of oocytes was investigated to determine its optimal concentration for IVM of oocytes. A total of 474 fresh oocytes were used in five replicates to evaluate the effects of melatonin during IVM of oocytes on their nuclear maturation to the metaphase-II (MII) stage. The rate of oocytes maturation to MII stage was significantly higher ($P < 0.05$) when matured in IVM medium supplemented with 10 ng/ml (73.2 ± 2.2%) and 50 ng/ml (67.2 ± 4.5) compared with oocytes matured in IVM medium with 0 ng/ml (the control; 49.1 ± 3.8%) or 100 ng/ml melatonin (51.2 ± 3.7; Table 1).

The Effect of Melatonin Supplementation During IVM on Cumulus Cell Expansion

As shown in Table 2, there was a significant ($P < 0.05$) increase in the percentage of oocytes having fully expanded cumulus cells in oocytes matured in the presence of 10 and 50 ng/ml of melatonin (60.5 ± 4.2% and 55.2 ± 4.9%, respectively) compared with control and 100 ng/ml melatonin-treated oocytes (38.6 ± 3.6% and 38.6 ± 3.0%, respectively).

The Effect of Melatonin Supplementation During IVM on COCs Steroidogenesis

The effect of melatonin supplementation to IVM medium on bovine COCs steriodogenic activity was assessed. After IVM of COCs for 22 hr in the absence or presence of 10, 50, and 100 ng/ml of melatonin, progesterone and estradiol concentrations were measured in the spent media (control, 0, 50, and 100 ng/ml medium groups, respectively; Table 3). Although a significant difference was not found in the levels of progesterone among the groups, there was a tendency for numerical increase, especially in the 10 and 50 ng/ml medium groups (48.9 ± 2.7 and 51.4 ± 3.5 ng/ml, respectively), compared with control and 100 ng/ml medium groups (44.8 ± 3.3 and 43.8 ± 2.3 ng/ml, respectively). Similarly, although the concentration of estradiol after maturation did not differ significantly among the groups, there was a tendency for reduction when the melatonin dose was increased; the highest average value was detected in the control group 1 (30.43 ± 10.25 pg/ml), whereas supplementation of IVM with 10, 50, and 100 ng/ml of melatonin showed a gradual reduction of this value (29.7 ± 6.5, 20.5 ± 7.1, and 11.08 ± 2.6 pg/ml, respectively).

The Effect of Melatonin Supplementation During IVM on the Activity and Distribution of Mitochondria in Oocytes

The effects of melatonin supplementation to IVM medium on the mitochondrial activity and distribution in bovine oocytes were investigated. There was no significant

TABLE 2. The Effect of Melatonin Supplementation on Bovine Cumulus Cell Expansion In Vitro*

Melatonin (ng/ml)	No. of oocytes examined	No (% mean ± SEM)** of oocytes with cumulus cells***		
		Not expanded	Partially expanded	Fully expanded
0	189	50 (26.4 ± 0.5)[a]	66 (34.9 ± 4.0)	73 (38.6 ± 3.6)[b]
10	170	19 (11.1 ± 2.2)[c]	48 (28.2 ± 3.3)	103 (60.5 ± 4.2)[a]
50	161	27 (16.7 ± 5.7)[bc]	45 (27.9 ± 1.8)	89 (55.2 ± 4.9)[a]
100	176	45 (25.5 ± 3.8)[ab]	63 (35.7 ± 2.6)	68 (38.6 ± 3.0)[b]

*The experiment was replicated four times.
**Percentage is based on the number of oocytes examined.
***After IVM, oocytes were classified according to the degree of cumulus expansion as not expanded, partially expanded (the outer layer of cells was loosened), or fully expanded (all cumulus cells were loosened).
[a-c]Values with different letters in the same column are significantly different ($P < 0.05$).

TABLE 3. The Effect of Melatonin Supplementation on Bovine Cumulus-Oocytes Complexes Steroidogenesis In Vitro

Duration of IVM (hr)	Supplementation of melatonin (ng/ml)	Estradiol (pg/ml)[a]	Progesterone (ng/ml)[a]
0	0	2.77 ± 0.71	0
22	0	30.42 ± 10.25	44.82 ± 3.36
22	10	29.77 ± 6.51	48.99 ± 2.77
22	50	20.54 ± 7.15	51.48 ± 3.58
22	100	11.08 ± 2.61	43.85 ± 2.34

Estradiol and progesterone measurements were replicated 9 and 13 times, respectively.
[a]Results are presented as mean ± SEM; significant difference between treatment groups was not detected ($P < 0.05$) by one-way ANOVA.

TABLE 5. The Effect of Melatonin Supplementation During In Vitro Maturation on Mitochondrial Distribution of Bovine Oocytes Matured to the Second Metaphase of the First Meiotic Division

Melatonin (ng/ml)	Total oocytes	Mitochondrial distribution type (% mean ± SEM)	
		Peripheral	Dispersed
0	54	57.5 ± 2.4[a]	42.4 ± 2.4[b]
10	60	33.3 ± 6.5[b]	66.6 ± 6.5[a]
50	55	28.0 ± 4.8[b]	71.9 ± 4.8[a]
100	55	50.9 ± 3.3[a]	49.0 ± 3.3[b]

Three trials were performed.
Results are presented as mean ± SEM.
Values with "a and b" are significantly different in the same column ($P < 0.05$).

difference in MitoTracker® Red CMXRos uptake among the oocytes as measured by the fluorescence intensity (Table 4). On the other hand, there was a significant difference in the distribution of active mitochondria in the oocyte cytoplasm ($P < 0.05$) among oocytes (Table 5 and Fig. 2). Significantly higher proportions of oocytes with dispersed mitochondria were observed in oocytes matured with 10 and 50 ng/ml melatonin (66.6% ± 6.5 and 71.9% ± 4.8, respectively) compared with oocytes matured with 0 (control, 42.4% ± 2.4) and 100 ng/ml melatonin (49.0% ± 3.3).

The Effect of Melatonin Supplementation During IVM on the Antioxidant Activity in Oocytes

As shown in Table 6, a total of 210 oocytes were used in five replications to estimate the effect of melatonin supplementation to IVM medium on the level of H_2O_2 in bovine oocytes. There was highly significant difference ($P < 0.05$) in H_2O_2 levels among oocytes, as measured by the relative fluorescence intensity. Significantly higher proportions of oocytes with high level of ROS were observed in oocytes matured with 0 (control, 100.00 ± 0.0) compared with oocytes matured with 10, 50, and 100 ng/ml melatonin (63.2 ± 5.2, 61.6 ± 5.3, and 62.3 ± 5.1, respectively). On the other hand, there was no significant difference in H_2O_2 levels among melatonin-treated groups.

TABLE 4. The Effect of Melatonin Supplementation on Mitochondrial Activity of Bovine Oocytes Matured In Vitro to the Second Metaphase of First Meiotic Division, Measured by the MitoTracker® Red CMXRos Uptake

Melatonin (ng/ml)	Area	Relative fluorescence values in oocytes after staining	
		Mean	Total[a]
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
10	105.0 ± 4.6	101.9 ± 3.2	106.9 ± 3.9
50	92.15 ± 7.4	100.5 ± 3.0	92.52 ± 6.4
100	109.2 ± 9.9	94.01 ± 2.6	101.5 ± 7.4

Eight trials were performed.
Results are presented as mean ± SEM; significant difference between treatment groups was not detected ($P < 0.05$) by one-way ANOVA.
[a]Total fluorescence in each oocyte was calculated as mean fluorescence - × oocyte (measurement) area.

DISCUSSION

Our study revealed, for the first time, the expression of specific melatonin receptor genes (*MTNR1A* and *MTNR1B*) and *ASMT* in bovine oocytes and cumulus cells. *MTNR1A* receptor expression was confirmed in oocytes and cumulus cells; *MTNR1B* was expressed in oocytes, but not in cumulus cells. Based on these findings it can be speculated that some effects of melatonin on bovine COCs are likely to be mediated by receptor mechanisms. Moreover, the expression of *MTNR1A* in oocytes and cumulus cells might confirm previous reports suggesting that *MTNR1A* is more important for regulation of reproduction than *MTNR1B* (Weaver

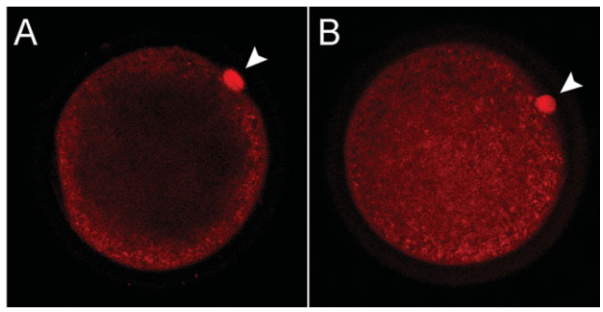

Figure 2. Mitochondrial distribution patterns in bovine IVM oocytes. **A**: An example of the peripheral location of active mitochondria in MII oocyte matured in IVM medium supplemented with 0 ng/ml of melatonin, indicating the distribution of mitochondria in the sub-cortical region with their absence in the central cytoplasm. **B**: An example of dispersed allocation of mitochondria in an MII oocyte matured in medium supplemented with 10 ng/ml of melatonin; mitochondria were distributed homogeneously and more centrally. Arrowheads indicate the first polar body. The images were taken by CLSM after staining with MitoTracker® Red CMXRos. Original magnification 400×.

TABLE 6. The Effect of Melatonin Supplementation During In Vitro Maturation of Bovine Oocytes on the Level of Reactive Oxygen Species (ROS), Measured by the 2′,7′-Dichlorofluoresceindiacetate (DCF) Fluorescence

Melatonin (ng/ml)	Relative total fluorescence values in oocytes after staining*
0	100.00 ± 0.00^{a}
10	63.2 ± 5.2^{b}
50	61.6 ± 5.3^{b}
100	62.3 ± 5.1^{b}

Five trials were performed.
Results are presented as mean ± SEM; a and b denote significant difference between treatment groups ($P < 0.05$) by one-way ANOVA.
*Total fluorescence in each oocyte was calculated as mean fluorescence × oocyte (measurement) area.

et al., 1996; Dubocovich et al., 1998). The expression of melatonin receptors in oocytes and cumulus cells implies that melatonin may act directly on bovine oocyte maturation via receptor mechanisms.

Also our study also revealed that *ASMT* was amplified from mRNA obtained from both bovine oocytes and cumulus cells. Melatonin is a small, lipophilic molecule derived from the amino acid tryptophan. Tryptophan is transformed into 5-hydroxytryptophan by a mitochondrial enzyme called tryptophan-5-hydroxylase (Lovenberg et al., 1967; Sitaram and Lees, 1978). 5-Hydroxytryptophan is, in turn, decarboxylated to serotonin by the cytoplasmic enzyme decarboxylase (Lovenberg et al., 1962). Another cytoplasmic enzyme called serotonin-acetyltransferase (NAT) completes the next step by transforming serotonin to N-acetyl serotonin (Weissbach et al., 1960). The final step in the pathway is the conversion of N-acetylserotonin by *ASMT* to melatonin (Wurtman and Axelrod, 1965). Recently, *ASMT* has been reported to be the new rate-limiting enzyme in melatonin biosynthesis (Ribelayga et al., 2000; Ceinos et al., 2004; Johnston et al., 2004; Liu and Borjigin, 2005; Reiter et al., 2007) instead of NAT (Klein and Weller, 1970). Thus, the expression of *ASMT* enzyme in bovine oocytes and cumulus cells in our study might confirm the previously suggested role of granulosa cells in melatonin biosynthesis (Itoh et al., 1999). Moreover, our results suggest that both oocyte and cumulus cells might play roles together in melatonin biosynthesis by unknown mechanisms.

Previously, melatonin has been the drug of choice for improving oocyte quality in women who cannot become pregnant due to poor oocyte quality (Takasaki et al., 2003). Increased melatonin concentration in the follicular fluid was reported to play a role in reducing the level of lipid peroxidation, which causes DNA damage in oocytes (Takasaki et al., 2003; Manjunatha et al., 2009). Also, melatonin was reported to enhance meiotic maturation of porcine (Kang et al., 2009a), buffalo (Manjunatha et al., 2009), and mouse oocytes in vitro (Ahn and Bae, 2004; Na et al., 2005). Moreover, melatonin supplementation to IVM medium is potent enough to release mouse oocytes from meiotic arrest by dbcAMP or hypoxanthine, regardless of the concentration of oxygen (Ahn and Bae, 2004; Na et al., 2005), suggesting its role in meiotic resumption. Our results showed, for the first time, that melatonin supplementation to IVM medium, especially at 10 and 50 ng/ml, greatly affected nuclear progression of bovine oocytes resulting in high frequency of MII oocytes after 22 hr of IVM. The high rates of control oocytes arrested at the MI stage suggest that melatonin may exert its positive effects on the progression from MI to MII stage by counteracting the free radical stressors in IVM medium or by unknown receptor-signaling mechanisms.

Multiple lines of evidence indicate that melatonin might act directly or synergistically with other hormones to alter granulosa and luteal cell steroidogenesis in vitro (Fiske et al., 1984; Webley and Luck, 1986; Webley et al., 1988; Brzezinski et al., 1991, 1992; Baratta and Tamanini, 1992; Woo et al., 2001; Tanavde and Maitra, 2003; Tamura et al., 2009). In the present study, there was no significant effect of melatonin supplementation during IVM on the ability of bovine COCs to produce progesterone. In accordance with our results, some previous reports showed no or even negative effects of melatonin on progesterone production in growing and luteinized GCs (Battista and Condon, 1986; Brzezinski et al., 1987; Khan-Dawood and Dawood, 1993; Sirotkin, 1994; Murayama et al., 1997; Schaeffer and Sirotkin, 1997; Tamura et al., 1998; Bódis et al., 2001; Nakamura et al., 2003). On the other hand, melatonin-stimulated secretion was reported in previous studies (Crisp and Channing, 1972; Tureck and Strauss, 1982; Sugino et al., 2000; Woo et al., 2001; Tamura et al., 2009; Taniguchi et al., 2009). Similarly, there was no significant effect of melatonin on estradiol-17β secretion by bovine COCs, although there was a tendency for its reduction with increasing melatonin concentration. Consistent with our results, melatonin failed to stimulate estradiol secretion after culturing of porcine antral follicles for 30 hr (Tanavde and Maitra, 2003). In contrast, melatonin-stimulated secretion was reported in a previous study on cultured granulosa cells of sow (Sirotkin, 1994), but no such effect was confirmed in sheep (Baratta and Tamanini, 1992) or rat cultured granulosa cells (Fiske et al., 1984). The direct effects of melatonin on sex steroid production remain poorly understood and seem to depend on different factors such as the cell type (theca cell or GC), experimental model (cell culture or follicle culture), species, dose and duration of treatment (Webley and Luck, 1986; Tamura et al., 2009; Adriaens et al., 2006).

Mammalian cumulus cells play a very important role during oocyte growth and maturation. They are known to supply nutrients (Eppig, 1982; Haghighat and Van Winkle, 1990; Laurincık et al., 1992) and/or messenger molecules for oocyte development (Lawrence et al., 1978; Thibault et al., 1987; Buccione et al., 1990), and to mediate the effects of hormones on oocytes (Zuelke and Brackett, 1990). Moreover, cumulus cell expansion is considered an important marker for oocyte maturation (Chen et al., 1990; Qian et al., 2003) and is essential for fertilization, subsequent cleavage, and blastocyst development (Gutnisky et al., 2007). Our results confirmed that melatonin supplementation to IVM medium had a potentially significant effect

on the degree of bovine cumulus cell expansion, especially at concentrations (10 and 50 ng/ml) proven to be optimal for nuclear maturation. The same promoting effects of melatonin on cumulus cell expansion were reported in porcine oocytes (Kang et al., 2009b). Nevertheless, it is not clear whether this enhancing effect was exerted via its receptors or its direct and indirect antioxidant activities. As an antioxidant, melatonin might protect cumulus cells against apoptosis (Sugino et al., 2000; Na et al., 2005; Taniguchi et al., 2009; Kang et al., 2009b) and enhance their expansion (Kang et al., 2009b).

It has been confirmed in previous studies that mitochondrial reorganization and metabolic activity are necessary features of cytoplasmic maturation and resumption of meiosis in oocytes (Van Blerkom and Runner, 1984; Hyttel et al., 1986, 1989; de Loos et al., 1989; Calarco, 1995; Van Blerkom et al., 1995; Cummins, 1998; Steeves and Gardner, 1999) that affect their subsequent development after fertilization (Van Blerkom and Runner, 1984; Calarco, 1995; Van Blerkom et al., 1995; Bavister and Squirrell, 2000). Our results indicated no major changes in the metabolism of mitochondria during maturation in oocytes treated with melatonin. On the other hand, mitochondrial distribution was modified during IVM in the presence of melatonin. Although several studies concerning the effect of melatonin on mitochondria in other cells types have been conducted, to our knowledge the effect of melatonin on bovine oocyte mitochondria has not been studied. Melatonin and its metabolites have been reported to have strong antioxidant capacity in vitro as well as in vivo to reduce lipid peroxidation and DNA damage (Reiter, 1991; Martin et al., 2000; Leon et al., 2005; Jou et al., 2007; Tan et al., 2007; Peyrot and Ducrocq, 2008). This potent antioxidant effect (Pieri et al., 1994; Livrea et al., 1997; Matuszak et al., 1997; Poeggeler et al., 2002) was reported to protect mitochondria from damage and aging (Bize et al., 1980), which otherwise reduce the membrane potential and increase the fragility of mitochondria (Cottrell et al., 2000; Dirks et al., 2006; Navarro and Boveris, 2007). Furthermore, melatonin acts as an anti-apoptotic hormone through down-regulation of mitochondrial cytochrome *c* release and inhibition of activation of caspase-9 and -3 (Baydas et al., 2005). Nanomolar concentrations of melatonin increased the mitochondrial electron transport chain activity and ATP production, and it has also been suggested that this compound increases mitochondrial membrane potential and thus counteracts the mechanisms involved in transition pore opening and cytochrome *c* release from mitochonidria (Martin et al., 2000; Acuna-Castroviejo et al., 2002). Although melatonin does not exert a direct effect upon the mitochondrial activity during IVM of bovine oocytes, our results suggest it might affect the machinery responsible for the movement of mitochondria, which is known to be mediated by microtubules in oocytes (Van Blerkom, 1991). Considering these facts, the proportion of oocytes having a dispersed distribution of mitochondria was higher when matured in IVM medium at concentrations proven to be optimal for nuclear maturation of oocytes; the dispersed distribution of mitochondria (microtubule organization) in bovine oocytes might be related to high rates of nuclear maturation. Further studies are needed to clarify the relationship between the mitochondrial distribution and nuclear maturation of bovine oocytes.

As shown in Table 6, the present study demonstrates that melatonin has a powerful antioxidative effect during IVM of bovine oocytes. Free radicals were generally thought to be harmful for embryonic development (Guérin et al., 2001). Melatonin was identified as a powerful, direct free radical scavenger (Hardeland et al., 1993, 1995; Allegra et al., 2003) that quenches the hydroxyl radical, superoxide anion radical, singlet oxygen, peroxyl radical, and the peroxynitrite anion (Reiter et al., 1998), and an indirect antioxidant via stimulating antioxidant enzymes (Reiter et al., 2000; Rodriquez et al., 2004). It stimulates the synthesis of glutathione (GSH) (Urata et al., 1999), augments the activities of other antioxidants (Gitto et al., 2001), like superoxide dismutase, glutathione peroxidase (GPx) glutathione reductase (GSR or GR), glucose-6-phosphate dehydrogenase, and inhibits nitric oxide synthase (Reiter et al., 1998). The ability of melatonin to regulate the GSH/GSSG balance by modulating enzyme activities appears to involve the action of melatonin at a nuclear binding site (Pablos et al., 1997). Moreover, its ability to alter the activity of antioxidative enzymes requires an interaction with either membrane or nuclear receptors (Reiter et al., 2003). Therefore, based upon the current results, we can conclude that melatonin supplementation during oocyte in vitro maturation was crucial for combating the oxidative stressors and their related damages directly or indirectly via its membrane receptors.

In conclusion, in our study the expression of melatonin receptor *MTNR1A* gene in both oocytes and their cumulus cells was observed while *MTNR1B* was expressed only in oocytes. Moreover, the expression of *ASMT* in both oocytes and cumulus cells suggests the role of these cells in melatonin biosynthesis. Furthermore, it was found that melatonin supplementation at concentrations of 10 and 50 ng/ml during IVM promotes nuclear transition from the MI to MII stage in IVM oocytes, resulting in a high nuclear maturation rate. The melatonin supplementation also resulted in increased rates of oocytes with dispersed mitochondria in the cytoplasm and expanded cumulus cells. The presence of melatonin receptors suggests the specific actions of this compound on these processes. Moreover, reduction of reactive oxygen species could be an aspect of the mechanism by which melatonin exerts its beneficial effects during bovine oocyte maturation.

MATERIALS AND METHODS

Oocyte Collection and In Vitro Maturation (IVM)

Bovine ovaries were transported from the slaughter house to the laboratory in sterile Dulbecco's phosphate buffered saline (D-PBS) at 37°C. They were washed in D-PBS and then in normal saline. Cumulus-oocyte complex (COCs) were aspirated from follicles ranging between 3 and 7 mm in diameter with an 18-gauge needle attached to a

TABLE 7. The List of Primers Used in This Study

Gene	Primer sequence	Gene accession no.
GAPDH	Forward: 5′-CTCCCAACGTGTCTGTTGTG-3′ Reverse: 5′-TGAGCTTGACAAAGTGGTCG-3′	NM_001034034.1
MTNR1A	Forward: 5′-AGCACGAATTCCCTCTGCTA-3′ Reverse: 5′-GAGCATCGGAACGATGAAAT-3′	XM_614283
MTNR1B	Forward: 5′-GTCTATGGGCTCCTGAACCA-3 Reverse: 5′-TCATTTTCCTGAGTGCGTTG-3′	XM_002698996.1
ASMT	Forward: 5′-CTCCCAGGTTCTCTTTGCTG-3′ Reverse: 5′-AGCTTCAGGGACACACAGGT-3′	NM_177493.2

disposable 10-ml syringe within 3–5 hr after the animal was slaughtered. Evenly granulated oocytes surrounded with multi-layered compact cumulus cells were selected for the experiments. Selected COCs were first washed three times in sterile D-PBS, and then washed three times in IVM medium; IVM was performed in TCM-199 without phenol red (Gibco BRL, Grand Island, NY) supplemented with 0.025 AU/ml porcine FSH (Denka Pharmaceutical, Kawasaki, Japan), 0.055 mg/ml sodium pyruvate (Wako Pure Chemical Industries, Osaka, Japan), 100 IU/ml penicillin G potassium (Meiji Seika, Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika) and 3 mg/ml bovine serum albumin (BSA) (Sigma, St. Louis, MO). The COCs were cultured in groups of 30–50/well using 10 μl IVM medium/oocyte in four well dishes (Nunclone™, Roskilde, Denmark) without mineral oil overlay for 22 hr at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Melatonin (M5250-250MG, lot No. 068k1538; Sigma-Aldrich, St. Louis, MO) stock solution (1,160 ng/ml) was prepared in TCM-199 without phenol red, filtered using 0.22-μm filters from Millipore Corporation (Millex® GV Co., Cork, Ireland), and kept at −4°C until use for a maximum of 12 days. In all experiments, melatonin was added to the IVM media at the concentrations of 0 (Control), 10, 50, or 100 ng/ml.

Assessment of Oocyte Nuclear Maturation

Oocyte maturation rate was examined on the basis of the presence of the first polar body and the nuclear morphology after 22 hr of IVM. In brief, oocytes were fixed for 24–48 hr in ethanol/acetic acid (3:1 v/v), stained with 1% orcein dissolved in 45% acetic acid, and then evaluated under a phase-contrast microscope (Nikon, Tokyo, Japan). Nuclear stages were distinguished by the morphology of chromatin material according to Hewitt et al. (1998). Oocytes with a second metaphase plate and first polar body were classified as matured at metaphase of the second meiotic cell division (MII).

Assessment of Cumulus Cell Expansion

The degree of cumulus cell expansion was subjectively assessed under a stereomicroscope after 22 hr of IVM; COCs were classified as not expanded, partially expanded (the outer layer of cells was loosened), or fully expanded (all cumulus cells were loosened) according to previous reports of Marei et al. (2009) and Marei et al. (2010).

RNA Isolation and RT-PCR

Fresh, immature oocytes were subjected to denudation by pipetting in 0.1% hyaluronidase enzyme in TCM-199 whereas matured ones were denuded by pipetting in TCM-199 medium only. Denuded oocytes and their respective cumulus cells were washed twice in D-PBS solution, and total RNA for each sample was isolated from 25 denuded oocytes and their corresponding cumulus cells using RNeasy Micro Kit (QIAGEN, Tokyo, Japan) with DNase I treatment according to the manufacturer's protocol. Complementary DNA was synthesized using a PrimeScript RT reagent Kit (TAKARA, Tokyo, Japan) by RT-PCR using specific primers for each gene (Table 7). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The conditions for PCR was run as follows: denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec for 25 cycles (*GAPDH*) and 35 cycles (*ASMT*, *MTNR1A*, and *MTNR1B*), with a final extension at 72°C for 7 min. The expression pattern was confirmed by 2% agarose gel electrophoresis.

Evaluation of Mitochondrial Characteristics in Oocytes

The activity and distribution of mitochondria in IVM oocytes were evaluated according to the method of Torner et al. (2004) with some modifications. This method is based on the uptake of MitoTracker® Red CMXRos dye (Molecular Probes, Eugene, OR), which selectively stains live mitochondria. According to the manufacturer's description, the accumulation of this stain depends on the mitochondrial membrane potential and its retention after fixation. Matured oocytes were denuded by pipetting in TCM-199 medium, and then incubated with 200 nM MitoTracker® Red CMXRos for 30 min in D-PBS containing 5% BSA under culture conditions. The oocytes were washed three times in pre-warmed D-PBS without BSA, and then fixed for 15 min at 37°C using 2% paraformaldehyde in D-PBS. After fixation, the oocytes were washed twice in D-PBS, mounted on slides under cover slips, and examined immediately at room temperature in a dark room. The fluorescence intensity was measured using an epifluorescent microscope (Nikon Eclipse E-600; Nikon). A grayscale photograph of 4–9 oocytes in each culture group was taken after excitation at a wavelength of 510–560 nm using a digital camera (Pixera Penguin 150 CL; Pixera Corp., Los Gatos, CA).

The mean fluorescence intensity and the surface area of each oocyte were measured in images using NIH Image J (v. 1.40) software (Abramoff et al., 2004). Total fluorescence was calculated as mean fluorescence (''mean gray value'') multiplied by oocyte surface (''area''). To express relative fluorescence the mean total fluorescence of the control group was considered to be 100%.

The distribution of active mitochondria was investigated qualitatively using a laser-scanning confocal microscope (Nikon D-eclipse C1) equipped with an argon–krypton–helium/neon ion laser and a 543 nm excitation barrier filter. To classify the distribution of active mitochondria, a cross-sectional image at the largest diameter of each oocyte was taken. Peripheral mitochondrial distribution was characterized by the location of active mitochondria beneath the plasma membrane (Fig. 2A). Diffuse distribution was characterized by the homogeneous location of mitochondria throughout the cytoplasm (Fig. 2B).

Measurement of H_2O_2 Contents

To determine the quantity of H_2O_2 produced by the oocytes that were matured in the presence or absence of melatonin for 22 hr under 5% CO_2 and 20% of O_2, the relative intensity of H_2O_2 production was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Sigma). DCHFDA is membrane permeant and, therefore, is able to diffuse readily into cells. Within the cell, acetate groups are hydrolyzed by intracellular esterase, forming 2′,7′-dichlorodihydrofluorescein (DCHF). DCHF then fluoresces when it is oxidized by H_2O_2 to 2′,7′-dichlorofluoresceindiacetate (DCF). The level of DCF produced within the cells is related to the concentration of hydrogen peroxide present; thus, its fluorescent emission enables measurement of the cellular hydrogen peroxide level (Nasr-Esfahani et al., 1990; Nasr-Esfahani and Johnson, 1991). Matured oocytes were denuded by pipetting in TCM-199 medium, and then incubated with 10 μm DCHFDA. After 15 min of incubation, oocytes were washed three times in new IVM medium, then mounted on slides under coverslips and examined immediately at room temperature in a dark room according to Karja et al. (2006). The fluorescence intensity was measured using an epifluorescent microscope (Nikon Eclipse E-600; Nikon). A grayscale photograph of 4–5 oocytes in each culture group was taken after excitation at a wavelength of 510–560 nm using a digital camera (Pixera Penguin 150 CL; Pixera Corp.). The mean fluorescence intensity and the surface area of each oocyte were measured in images using NIH Image J (v. 1.40) software (Abramoff et al., 2004). Total fluorescence was calculated as mean fluorescence (''mean gray value'') multiplied by oocyte surface (''area''). To express relative fluorescence the mean total fluorescence of the control group was considered to be 100%.

Hormone Measurements

Estradiol and progesterone concentrations in the culture media before and after IVM were measured with commercially available radioimmunoassay kits (ESTR-CTRIA and PROG-CTRIA, respectively, CIS Bio International, Gif-Sur-Yvette, France) according to the attached protocols. Media were directly applied for the estradiol assay, but were diluted five times with distilled water before the progesterone assay.

Statistical Analysis

Statistical analyses were carried out by Graph Pad Prism software 2007 version 5.03 (Graph Pad Prism, San Diego, CA) for determining the significant difference between treatment groups by one-way analysis of variance (ANOVA). Paired and unpaired Student *t* tests were used to detect the significance of difference between pairs of groups. Data were represented as percentage of mean ± SEM. Significant difference between mean values was determined at $P < 0.05$.

Experimental Design

Experiment 1 The expressions of melatonin receptor genes *MTNR1A*, *MTNR1B*, and *ASMT* were assessed in oocytes and cumulus cells before and after IVM with melatonin at various concentrations (10, 50, and 100 ng/ml) or no addition (Control) in six replications.

Experiment 2 The effects of melatonin supplementation (10, 50, or 100 ng/ml) during IVM on cumulus cell expansion and nuclear maturation of bovine oocytes were investigated; IVM without melatonin supplementation served as a control. After IVM, the expansion of cumulus cells was assessed, and then the nuclear stages of the oocytes were compared. The experiments were replicated at least four times.

Experiment 3 The effect of melatonin supplementation (10, 50, or 100 ng/ml) during IVM on steroidogenesis in COCs was investigated; IVM without melatonin supplementation served as a control. Estradiol and progesterone concentrations were measured in the spent media before and after IVM.

Experiment 4 The effect of melatonin supplementation during IVM on the activity and distribution of mitochondria in oocytes was investigated. The activity and distribution of mitochondria were evaluated using oocytes matured in the absence (Control) or presence of 10, 50, or 100 ng/ml of melatonin. The experiments were replicated eight times to assess mitochondrial activity and three times to assess mitochondrial distribution.

Experiment 5 The effect of melatonin supplementation (10, 50, or 100 ng/ml) during IVM on the level of reactive oxygen species was investigated; IVM without melatonin supplementation served as a control. After IVM denuded oocytes were subjected to DCHFDA, mounted on glass

slides and examined by epifluorescent microscope. The experiment was replicated five times.

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