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Melatonin enhances the *in vitro* maturation and developmental potential of bovine oocytes denuded of the cumulus oophorus

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Summary

This study was designed to determine the effect of melatonin on the *in vitro* maturation (IVM) and developmental potential of bovine oocytes denuded of the cumulus oophorus (DOs). DOs were cultured alone (DOs) or with 10^{-9} M melatonin (DOs + MT), cumulus–oocyte complexes (COCs) were cultured without melatonin as the control. After IVM, meiosis II (MII) rates of DOs, and reactive oxygen species (ROS) levels, apoptotic rates and parthenogenetic blastocyst rates of MII oocytes were determined. The relative expression of ATP synthase F0 Subunit 6 and 8 (*ATP6* and *ATP8*), bone morphogenetic protein 15 (*BMP-15*) and growth differentiation factor 9 (*GDF-9*) mRNA in MII oocytes and IFN-tau (*IFN-τ*), *Na^+/K^+-ATPase*, catenin-beta like 1 (*CTNNBL1*) and *AQP3* mRNA in parthenogenetic blastocysts were quantified using real-time polymerase chain reaction (PCR). The results showed that: (1) melatonin significantly increased the MII rate of DOs (65.67 ± 3.59 % vs. 82.29 ± 3.92%; $P < 0.05$), decreased the ROS level (4.83 ± 0.42 counts per second (c.p.s) vs. 3.78 ± 0.29 c.p.s; $P < 0.05$) and apoptotic rate (36.99 ± 3.62 % vs. 21.88 ± 2.08 %; $P < 0.05$) and moderated the reduction of relative mRNA levels of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* caused by oocyte denudation; (2) melatonin significantly increased the developmental rate (24.17 ± 3.54 % vs. 35.26 ± 4.87%; $P < 0.05$), and expression levels of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* mRNA of blastocyst. These results indicated that melatonin significantly improved the IVM quality of DOs, leading to an increased parthenogenetic blastocyst formation rate and quality.

Keywords: Bovine, Denuded oocytes, Development potential, *In vitro* maturation, Melatonin

Introduction

Melatonin (*N*-acetyl-5-hydroxytyrptamine) is an indole found in vertebrates (Stehle *et al.*, 2011), which modulates circadian and circannual rhythms such as the sleep/wake cycle and seasonal reproduction (Lincoln *et al.*, 2006; Reiter *et al.*, 2010). Additionally, melatonin and its metabolites are potent free radical scavengers and antioxidants (Chen *et al.*, 2006; Manda *et al.*, 2007). In recent years, researchers have begun to pay attention to the *in vitro* and *in vivo* effects of melatonin on the maturation of oocytes and development of mammalian embryos (Berlinguer *et al.*, 2009; Shi *et al.*, 2009).

High levels of melatonin have been detected in human (Brzezinski *et al.*, 1987; Rönnberg *et al.*, 1990) and porcine follicular fluid (Shi *et al.*, 2009), suggesting that melatonin may influence mammalian ovarian and reproductive function. The fertilization rate of retrieved oocytes, compared with the previous *in vitro*

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fertilization and embryos transfer (IVF-ET) cycle, was increased significantly when patients were administered 3 mg/day melatonin (Tamura *et al.*, 2008), and melatonin implants could significantly increase the developmental abilities of goat (Berlinguer *et al.*, 2009) and ovine (Vázquez *et al.*, 2010) oocytes after IVF.

As a free radical scavenger, antioxidant and anti-apoptotic agent (Chen *et al.*, 2006), melatonin has been added to culture medium to improve the development of mouse (Ishizuka *et al.*, 2000), porcine (Rodriguez-Osorio *et al.*, 2007) and bovine (Papis *et al.*, 2007) embryos. Meanwhile, Manjunatha *et al.* (2009) reported that supplementation of *in vitro* maturation (IVM) medium for cumulus–oocyte complexes (COCs) with melatonin improved *in vitro* embryo production efficiency in buffalo, with a high transferable embryo yield obtained by enriching culture medium with 10 μM melatonin. Optimal cleavage (79%) and blastocyst rates (35%) were obtained when 10^{-9} M melatonin was added to both the IVM medium of porcine COCs and the culture medium for parthenogenetic embryos (Shi *et al.*, 2009). Furthermore, melatonin supplementation during IVM of COCs resulted in a greater proportion of oocytes extruding the polar body and ROS levels of melatonin-treated oocytes were significantly lower than untreated oocytes in porcine (Kang *et al.*, 2009) and bovine (El-Raey *et al.*, 2011). However, little information is available about the approaches via which melatonin promotes the maturation of oocytes.

Therefore, to investigate the direct effect of melatonin on oocyte maturation and eliminate the ability of cumulus cells to affect oocyte maturation, denuded oocytes (DOs) were used as a research model in our study. The effect of melatonin on the metaphase-II (MII) rate of DOs was assessed, compared with DOs and COCs in control media. ROS levels, the apoptotic rates and expression levels of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* were determined in MII oocytes. Additionally, the parthenogenetic blastocyst rate and expression levels of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* were quantified in parthenogenetic blastocysts to assess the effect of melatonin on the formation rate and quality of parthenogenetic blastocysts.

Materials and methods

Unless otherwise indicated, chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO, USA). The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals

Table 1 Effect of concentrations of melatonin on the nuclear maturation of oocytes denuded of the cumulus oophorus (DOs)

Groups	No. DOs cultured	No. MII oocytes (%)
DOs + 10^{-7} M MT	119	89 (74.79 ± 5.37%)^{a,b}
DOs + 10^{-9} M MT	128	103 (80.47 ± 6.95%)^{a}
DOs + 10^{-11} M MT	135	102 (75.56 ± 7.18%)^{a,b}
DOs	100	66 (66 ± 4.63%)^{b}
COCs	117	99 (84.62 8.82%)^{a}

^{a,b,c}Values with different superscripts indicate significant difference within the same column ($P < 0.05$).
MT, melatonin.

Oocytes collection and IVM

Bovine ovaries were collected from the local abattoir and transported to the laboratory within 2 h. COCs were aspirated from follicles 2–8 mm in diameter and those with at least three layers of compact cumulus cells were used for IVM. To obtain DOs, COCs were denuded of cumulus cells by vortexing in 0.1% (w/v) hyaluronidase for 2–3 min.

Groups of 50 COCs or DOs were cultured in four-well dishes in an incubator at 38.5°C with 5% CO_2. IVM was performed for 22–24 h in 500 μl IVM medium that contained medium 199 (Gibco BRL, Carlsbad, CA, USA) supplemented with 10 μg/ml follicle stimulating hormone (FSH), 10 μg/ml luteinising hormone (LH), 10% (v/v) fetal bovine serum (FBS, HyClone; Gibco BRL), 10 μg/ml estradiol and 10 μg/ml heparin.

In our preliminary study, the IVM medium of DOs was supplemented with 10^{-7} M to 10^{-11} M melatonin and the highest MII rate of DOs was achieved with 10^{-9} M melatonin (Table 1), so 10^{-9} M melatonin was supplemented in the DOs + MT group in this experiment.

Assessment of oocyte maturation

The MII oocyte phase was determined by evaluating the presence of the first polar body, according to the method described by Kang *et al.* (2009) with some modifications. After 22–24 h IVM, DOs were fixed in methanol for 10 min, mounted on a slide, stained with 10 μg/ml Hoechst 33342 and the presence or absence of polar bodies was determined by a fluorescence microscopy (Olympus IX70; Olympus, Tokyo, Japan), as shown in Fig. 1.

Analysis of ROS Levels in MII oocytes

The ROS levels in MII oocytes were measured according to the method described by Rahimi *et al.* (2003) with some modifications. The intracellular

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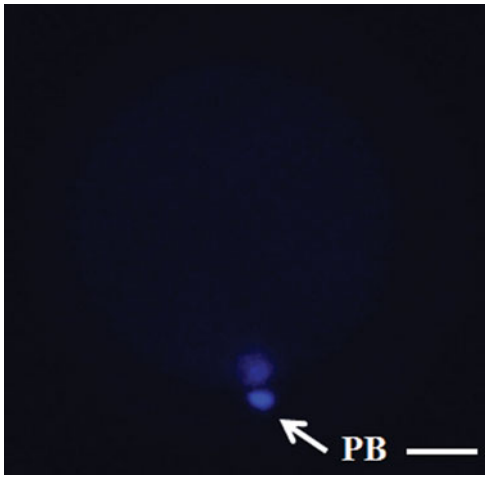

Figure 1 Nuclear staining of bovine oocytes after IVM. PB: the first polar body. Bars = 20 μm.

redox state was measured using the fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA; Genmed Scientifics, Inc., Arlington, MA, USA).

Briefly, 10 to 20 oocytes were washed three times in washing solution, transferred to 50 μl working solution containing dye and dilution buffer (in a ratio of 1:200) and stained for 20 min in an incubator at 38.5°C with 5% CO_2. The oocytes were washed three times in storage solution, transferred into 96-well dishes that contained 100 μl pre-warmed storage solution per well and fluorescence was immediately measured using a luminometer (Infinite M200; Tecan Group Ltd., Untersbergstrasse, Austria) at fluorescence excitation and emission wavelengths of at 488 nm and 530 nm, respectively. The ROS level per oocyte was calculated by dividing the total ROS level by the number of oocytes in each sample and expressed as photon counts per second (c.p.s).

Quantification of the apoptotic rate of MII oocytes using the annexin-V assay

MII oocytes were stained with annexin-V–fluorescein isothiocyanate (FITC) reagent (Biovision, Mountain View, CA, USA) in accordance with the manufacturer's instructions. Briefly, MII oocytes were washed three times in phosphate-buffered saline (PBS), incubated in 500 μl binding buffer containing 1 μl annexin-V–FITC and 1 μl propidium iodide (PI) for 5–10 min at room temperature in the dark, mounted and examined with a fluorescence microscope (Olympus) equipped with a CoolSNAP HQ CCD (Photometrics/Roper Scientific, Inc., Tucson, AZ, USA). According to the method described by Anguita *et al.* (2007), bovine DOs were classified into three groups after annexin-V staining, representing: (1) early apoptotic oocytes with a homogeneous annexin positive signal in the membrane (Fig. 2*A*); (2) viable oocytes no annexin staining (Fig. 2*B*); and (3) necrotic oocytes which showed PI-positive red nuclei (Fig. 2*C*).

Quantification of the relative expression of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* mRNA in MII oocytes

Total RNA was extracted from 100 MII oocytes from the COCs, DOs and DOs + MT groups using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The RNA pellet was dissolved in sterile water, RNA concentration was measured using a Beckman DU® 640 spectrophotometer (Beckman, Fullerton, CA, USA) and cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Briefly, 2 μg total RNA was mixed with 5 mM random hexamers and 8 μl diethylpyrocarbonate (DEPC) water, incubated at 70°C for 5 min to denature secondary structure and cooled rapidly to 0°C. Then 10 μl 5× RT buffer, 250 mM dNTPs, 40 U RNase inhibitor (Promega) and 400 U M-MLV reverse transcriptase were added to a total volume of 20 μl. The mixture was incubated at 50°C for 60 min, then at 95°C for 5 min to inhibit RNase activity and treated with RNase-free DNase (Promega).

The PCR primers for each gene were listed in Table 2. PCR reactions were carried out in a total volume of 25 μl, that contained 1.2 μl cDNA, 0.5 μl (10 μM) each primer and 0.5 μl 20× Master SYBR Green mix (Invitrogen) at 95°C for 2 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30s. Quantitative real-time PCR was performed on the ABI7500 SDS (Applied Biosystems, Foster, CA, USA) using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen & Livak, 2008) with β-*actin* as a reference gene.

Parthenogenetic activation

MII oocytes were treated with 5 μM of the Ca-ionophore A23187 for 5 min, then incubated in 2 μM 6-dimethylaminopurine (6-DMAP) for 4 h. After activation, oocytes were cultured in 100 μl CR1aa (Rosenkrans & First, 1994) supplemented with 0.1% (w/v) bovine serum albumin (BSA) under mineral oil for 48 h in 35 × 10 mm culture dishes (FALCON, NJ, USA) in an incubator at 38.5ºC with 5% CO_2, then cultured for 5 days in CR1aa supplemented with 10% (v/v) FBS, changing the medium every 2 days.

Total nuclear counts

Blastocysts were stained with Hoechst 33342 as mentioned above. Then they were viewed under ultraviolet light, and the total nuclear number of

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Table 2 Primers sequences, expected fragment sizes of detected genes of MII oocytes

Gene	Primers sequences (5′–3′)	Size (bp)	GenBank accession no.	Annealing temperature (°C)
ATP6	F:GAACACCCACTCCACTAATCCCAAT R:GTGCAAGTGTAGCTCCTCCGATT	147	AF493542	60
ATP8	F:CACAATCCAGAACTGACACCAACAA R:CGATAAGGGTTACGAGAGGGAGAC	129	AF493542	60
BMP-15	F:GAGGCTCCTGGCACATACAGAC R:CTCCACATGGCAGGAGAGGT	134	NM_001031752.1	60
GDF-9	F:CAGAAGCCACCTCTACAACACTG R: CTGATGGAAGGGTTCCTGCTG	95	NM_174681.2	60
β-*Actin*	F:CCTCCATCGTCCACCGCAAAT R:GCTGTCACCTTCACCGTTCCA	199	NM_173979	60

F, Forward primer; R, reverse primer.

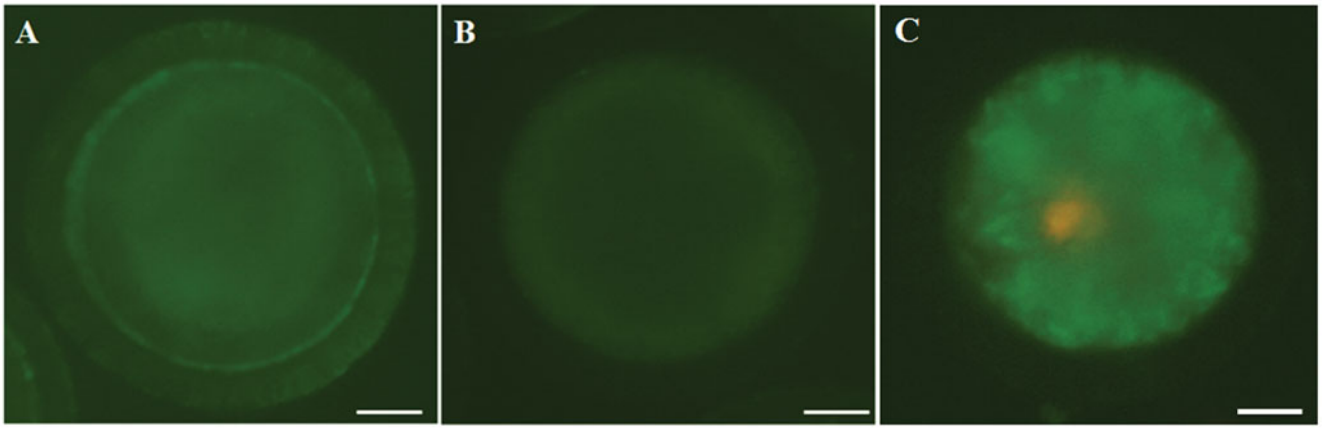

Figure 2 Classification of annexin-V stained bovine oocytes after IVM. (*A*) Annexin-V positive: a clear green signal was observed in the cytoplasmic membrane. (*B*) Annexin-V negative: no signal was observed in the cytoplasmic membrane. (*C*) Necrotic oocyte: Propidium iodide (PI)-positive nucleus was observed. Bars = 20 μm.

every blastocyst was counted under fluorescence microscopy.

Quantification of relative expression of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* mRNA in blastocysts

RNA was isolated from pools of 60 blastocysts from the COCs, DOs and DOs + MT groups. RNA isolation, reverse transcription and quantitative real-time PCR were performed as described previously, using the primers listed in Table 3.

Statistical analysis

All results were presented as mean ± standard error and each experiment was repeated at least three times. All percentage data were subjected to arcsine transformation before statistical analysis using Statistical Analysis System (SAS) software (SAS Institute; Cary, NC, USA). One-way analysis of variance (ANOVA) was used to determine significant differences in data levels, and Duncan's test was followed to determine statistical differences between groups. *P*-values < 0.05 were considered statistically significant.

Results

The effect of melatonin on the nuclear maturation of bovine DOs

As shown in Table 4, the MII rate of the DOs + MT group (82.29 ± 3.92%) was significantly higher than the DOs group (65.67 ± 3.59%; $P < 0.05$) and similar to the COCs group (85.33 ± 8.84%; $P > 0.05$).

The effect of melatonin on the ROS level of MII oocytes

The ROS level of DOs + MT group (3.78 ± 0.29 c.p.s) was significantly lower than that of the DOs group

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Table 3 Primer sequences, expected fragment sizes of detected genes of blastocysts

Gene	Primers (5′–3′)	Size (bp)	GenBank accession no.	Annealing temperature (°C)
IFN-τ	F: GCTCCAGAAGGATCAGGCTATC R: TGTTCCAAGCAGCAGACGAGT	95	AF238611	60
Na^+/K^+-ATPase	F: GCAGCAGTGGACCTATGAACAG R: CCAGGGCAGTAGGAAAGGAAAG	210	X02813	60
CTNNBL1	F:GTTCCTGCCTAATGCTGAGTTCC R:GGTCCGTAAGCCAAGAATGTCA	191	NM_174637.3	60
AQP3	F: AACCCTGCTGTGACCTTTGCTA R: TTGACCATGTCCAAGTGTCCAG	230	AF123316	60
β-*actin*	F: TCCTGGGCATGGAATCCTG R: GGCGCGATGATCTTGATCT	199	NM_173979	60

F, Forward primer; R, reverse primer.

Table 4. Effect of melatonin on the MII rate of bovine oocytes denuded of the cumulus oophorus

Groups	No. of oocytes	No. of MII oocytes (%)
COCs	1500	1280 (85.33 ± 8.84)^a
DOs + MT	2100	1728 (82.29 ± 3.92)^a
DOs	2400	1576 (65.67 ± 3.59)^b

^{a,b}Values with different superscripts indicate significant difference within the same column ($P < 0.05$).
COCs, cumulus–oocyte complexes; DOs, oocytes denuded of the cumulus oophorus; MT, melatonin.

Table 5 Effect of melatonin on the reactive oxygen species (ROS) levels of MII oocytes

Groups	No. of MII oocytes	ROS level (c.p.s/oocyte)
COCs	90	2.95 ± 0.13^c
DOs + MT	120	3.78 ± 0.29^b
DOs	60	4.83 ± 0.42^a

^{a,b,c}Values with different superscripts indicate significant difference within the same column ($P < 0.05$).
COCs, cumulus–oocyte complexes; DOs, oocytes denuded of the cumulus oophorus; MT, melatonin.

(4.83 ± 0.42 c.p.s; $P < 0.05$) and higher than the COCs group (2.95 ± 0.13 c.p.s; $P < 0.05$; Table 5).

The effect of melatonin on the apoptotic rate of MII oocytes

Representative positive and negative annexin-V staining images were presented in Fig. 2. The apoptotic rate of DOs + MT group (21.88 ± 2.08%) was significantly lower than the DOs group (36.99 ± 3.62%; $P < 0.05$) and higher than the COCs group (12.94 ± 0.83%; $P < 0.05$; Fig. 3).

The effect of melatonin on expression of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* mRNA in MII oocytes

The relative expression levels of *ATP6* and *ATP8* mRNA in MII oocytes from DOs + MT group were significantly higher than the DOs group ($P < 0.05$) and lower than the COCs group ($P < 0.05$). Significant differences of *BMP-15* and *GDF-9* mRNA expression levels were also observed in the DOs + MT, DOs and COCs groups ($P < 0.05$; Fig. 4).

The effect of melatonin on the developmental potential in MII oocytes

As shown in Table 6, there was no significant difference in the cleavage rates of the COCs (91.30 ± 6.08%), DOs + MT (92.42 ± 8.21%) and DOs groups (92.66 ± 5.53%; $P > 0.05$). However, the blastocyst rate of the DOs MT group (35.26 ± 4.87%) was significantly higher than the DOs group (24.17 ± 3.54%; $P < 0.05$) and lower than the COCs group (46.32 ± 4.29%; $P < 0.05$). The cell number of the DOs + MT group (74.92 ± 5.82) was significantly higher than that of the DOs group (52.74 ± 6.13; $P < 0.05$), and similar to that of COCs group (78.25 ± 8.37; $P > 0.05$).

The effect of melatonin on the expression of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* in parthenogenetic blastocysts

The relative expression level of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* in blastocysts from the DOs + MT group were significantly higher than those from the DOs group ($P < 0.05$; Fig. 5). The relative expression levels of *IFN-τ* and *Na^+/K^+-ATPase* in blastocysts from the DOs + MT group were significantly lower than those from COCs group ($P < 0.05$). The relative expression levels of *CTNNBL1* in blastocysts from DOs + MT group and COCs groups were similar ($P > 0.05$), and *AQP3* expression level in blastocysts from the DOs + MT group was

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Table 6 Effect of melatonin on the developmental potential of MII oocytes

Groups	No. of MII oocytes	No. of cleaved oocytes (%)	No. of blastocysts (%)	No. of cells/blastocyst
COCs	759	693 (91.30 ± 6.08)	321(46.32 ± 4.29)^{a}	78.25 ± 8.37 ($n = 30$)^{a}
DOs + MT	1068	987 (92.42 ± 8.21)	348 (35.26 ± 4.87)^{b}	74.92 ± 5.82 ($n = 30$)^{a}
DOs	1036	960 (92.66 ± 5.53)	232 (24.17 ± 3.54)^{c}	52.74 ± 6.13 ($n = 30$)^{b}

^{a,b,c}Values with different superscripts indicate significant difference within the same column ($P < 0.05$).
COCs, cumulus–oocyte complexes; DOs, oocytes denuded of the cumulus oophorus; MT, melatonin.

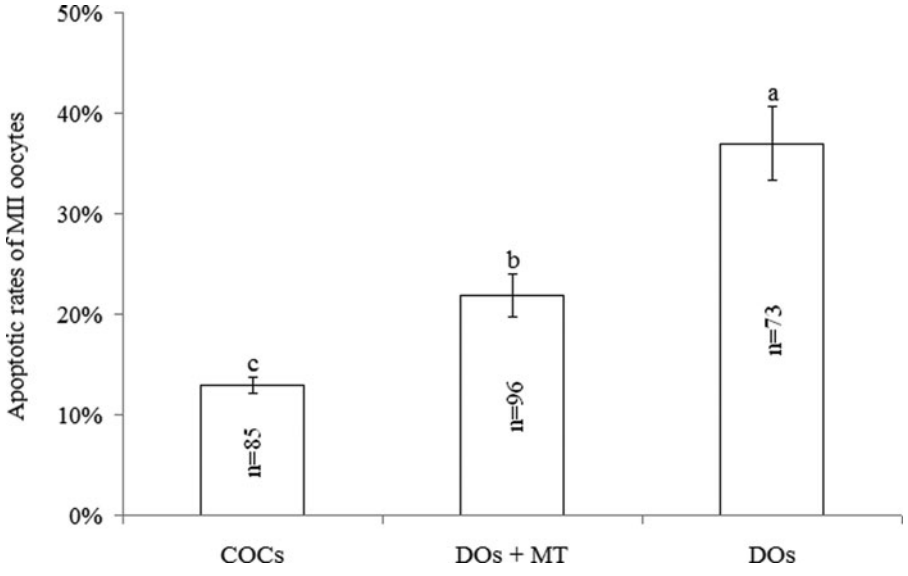

Figure 3 Apoptotic rates of MII oocytes of COCs, DOs + MT and DOs groups. ^{a,b,c}Values with different superscripts indicate significant difference ($P < 0.05$). COCs, cumulus–oocyte complexes; DOs, oocytes denuded of the cumulus oophorus; MT, melatonin.

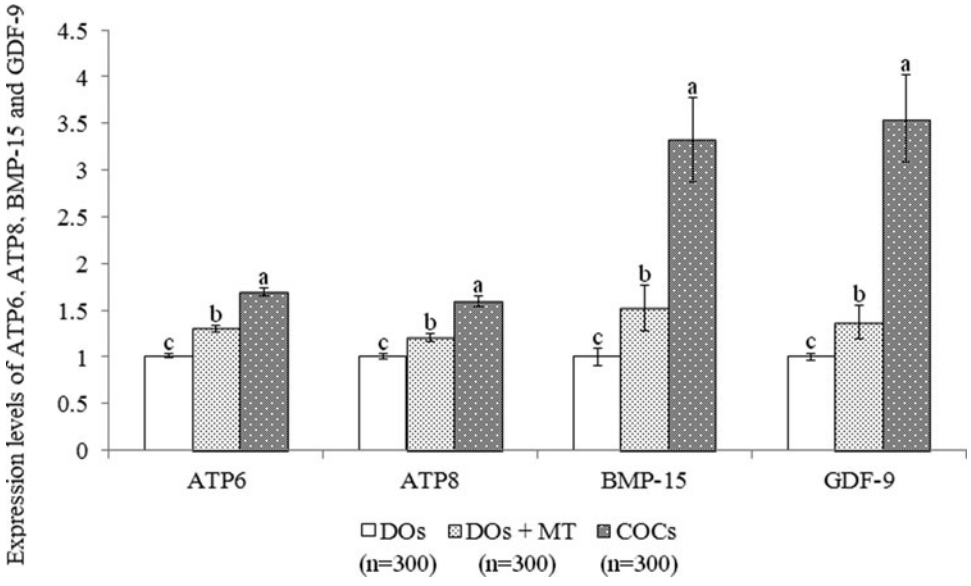

Figure 4 Relative expression levels of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* in MII oocytes. ^{a,b,c}Values of different superscripts indicate significant difference within the expression level of each gene ($P < 0.05$). COCs, cumulus–oocyte complexes; DOs, oocytes denuded of the cumulus oophorus; MT, melatonin.

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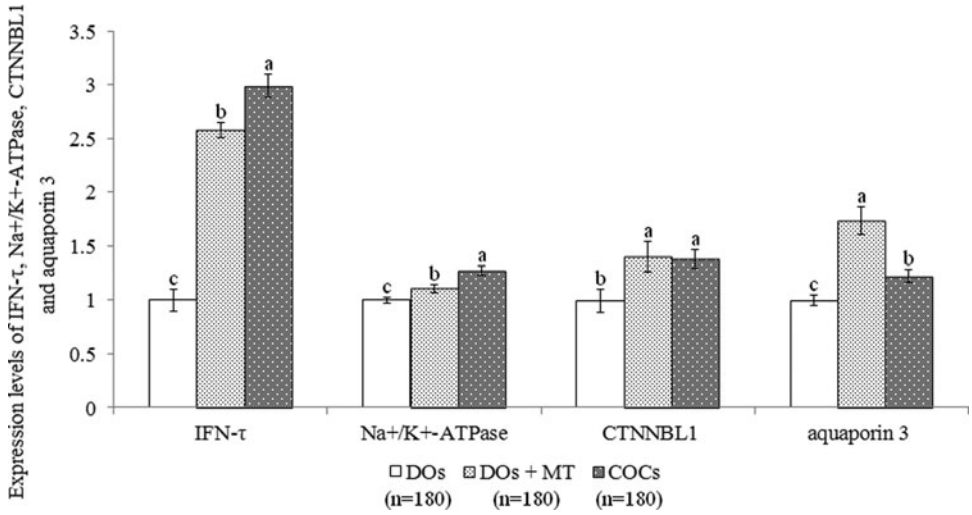

Figure 5 Relative expression levels of *IFN-τ*, *Na⁺/K⁺-ATPase*, *CTNNBL1* and *AQP3* in parthenogenetic blastocysts. [a,b,c]Values of different superscripts indicate significant difference within the expression level of each gene ($P < 0.05$). COCs, cumulus–oocyte complexes; DOs, oocytes denuded of the cumulus oophorus; MT, melatonin.

significantly higher than those from the COCs group ($P < 0.05$).

Discussion

Melatonin increased the nuclear maturation of bovine DOs

The removal of cumulus cells significantly decreased the MII rate from 85.33 ± 8.84% to 65.67 ± 3.59% ($P < 0.05$; Table 4), similar to the effects observed in bovine (Zhang *et al.*, 1995; Geshi *et al.*, 2000), porcine (Maedomari *et al.*, 2007) and mouse (Ge *et al.*, 2008a,b) oocytes. However, 10^{-9} M melatonin significantly increased the MII rate of DOs from 65.67 ± 3.59% to 82.29 ± 3.92% in the present study ($P < 0.05$).

It has been reported that melatonin acts via three distinct mechanisms of action: receptor-mediated, protein-mediated and non-receptor-mediated effects and the receptor-mediated action of melatonin involves both membrane and nuclear melatonin binding sites (Acuña-Castroviejo *et al.*, 1994, 2001). Two distinct subtypes of the melatonin receptor (melatonin receptor 1A (MTNR1A) and melatonin receptor 1B (MTNR1B)) have been cloned and mapped in several animal species (Messer *et al.*, 1997; von Gall *et al.*, 2002). MTNR1A is more strongly associated with reproductive activity than MTNR1B (Weaver *et al.*, 1996). MTNR1A is detected in bovine oocytes and cumulus cells, whereas MTNR1B is expressed in bovine oocytes but not bovine cumulus cells (El-Raey *et al.*, 2011). As we removed the cumulus cells from oocytes in our experiments and both MTNR1A and MTNR1B can be detected in bovine oocytes (El-Raey *et al.*, 2011), it was possible that melatonin directly promoted the MII rate of bovine DOs via its receptors on the oocyte membrane. Additionally, melatonin also accelerated the formation of maturation-promoting factor, which could improve the nuclear maturation of oocytes (Shi *et al.*, 2009).

Melatonin reduced ROS levels of MII oocytes

Cumulus cells were linked to oocytes by gap junction communications, via which cumulus cells regulated the synthesis of glutathione (GSH) (de Matos *et al.*, 1997) to maintain the redox state in cells and protect them against harmful effects caused by oxidative injuries (Meister, 1983; Gasparrini *et al.*, 2006). The GSH content is significantly lower in DOs than in cumulus-enclosed oocytes in cattle (de Matos *et al.*, 1997) and pigs (Cui *et al.*, 2009). The ability of COCs to protect against the induction of oxidative stress in oocytes may explain the significantly higher ROS levels observed in DOs group (4.83 ± 0.42 c.p.s) than COCs group (2.95 ± 0.13 c.p.s; Table 5) in this study.

Melatonin was reported to influence the activity and cellular mRNA expression levels of antioxidant enzyme enzymes in neuronal cell lines (Mayo *et al.*, 2002) and human oocytes (Tamura *et al.*, 2008). Superoxide dismutase activities were increased in the liver (Ozturk *et al.*, 2000), kidney and brain (Liu & Ng, 2000) of melatonin-treated rats. Metabolites of melatonin are likewise excellent scavengers of ROS (Manda *et al.*, 2007). All this evidence helped to explain the decreased ROS level in DOs + MT group (3.78 ± 0.29 c.p.s) compared with the DOs group (4.83 ± 0.42 c.p.s), similar to the observations in porcine (Kang *et al.*, 2009) and bovine COCs (El-Raey *et al.*, 2011).

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The presence of antioxidant enzyme transcripts at the germinal vesicle/MII stage in mouse oocytes and MII stage in human oocytes (El Mouatassim *et al.*, 1999) suggested that antioxidant defence mechanism were important for further oocyte maturation (Tamura *et al.*, 2008). Moreover, Tamura *et al.* (2008) reported that incubation of mouse oocytes with 300 μM H_2O_2 for 12 h significantly reduced the percentage of MII oocytes. In this study, a lower MII rate was observed in DOs group with a higher ROS level compared with DOs + MT group, indicating that high ROS levels could negatively influence oocyte nuclear maturation as discussed above. It was inferred from these results that melatonin may influence the MII rate of bovine DOs via a non-receptor-mediated mechanisms linked altered ROS accumulation besides the receptor-mediated approach mentioned above.

Melatonin decreased the apoptotic rates of MII oocytes

Externalization of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane occurs when apoptosis is activated (Lahorte *et al.*, 2004) and can be detected using the annexin-V assay (Chan *et al.*, 1998). The annexin-V assay could be used to detect the initiation of apoptosis at the MII stage in bovine oocytes and the rate of first cleavage and subsequent embryonic development of oocytes during IVF decreased with annexin-V binding increasing (Kalo & Roth, 2011).

Chen *et al.* (2006) reported that 5 mg/kg melatonin-treated mice had reduced cellular shrinkage and chromatin condensation in boundary zones of ischemic infarct, suggesting melatonin had an anti-apoptotic effect and could counteract post-ischemic radical-mediated apoptosis in the neurovascular unit. Similarly, our experiments demonstrated that melatonin significantly decreased the apoptotic rate of oocytes from 36.99 ± 3.62% to 21.88 ± 2.08% during IVM (Fig. 3). The lower apoptotic rate of DOs + MT group compared with the DOs group may be due to the ability of melatonin to act as a potent free radical scavenger and antioxidant (Chen *et al.*, 2006) and/or the inhibition of apoptosis by melatonin via MTNR1A or MTNR1B receptor-mediated mechanisms (Lanoix *et al.*, 2012).

The lower rate of apoptosis observed in the COCs group (12.94 ± 0.83%) compared with the DOs (36.99 ± 3.62%) and DOs + MT (21.88 ± 2.08%) groups may be due partially to the decreased ROS levels, as the higher levels of ROS observed in DOs and DOs + MT groups may activate a cascade of molecular event, leading to apoptosis (Simon *et al.*, 2000; Shimizu *et al.*, 2004).

Melatonin increased the expression of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* in MII oocytes

The expression of mitochondrial genes is known to affect the quality, fertilization and embryo development of oocytes (Hsieh *et al.*, 2004). Also, the expression of *GDF-9* and *BMP-15* is essential for the development and function of mouse (Su *et al.*, 2004) and human (Wei *et al.*, 2011) oocytes, and supplementation of exogenous BMP-15 or GDF-9 during the IVM significantly increased the development potential of bovine (Hussein *et al.*, 2006) and mouse (Yeo *et al.*, 2008) oocytes. Based on the above-mentioned finding, the expression levels of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* mRNA could be used to assess the oocytes quality.

This study showed that during the IVM of DOs, 10^{-9} M melatonin significantly moderated the reduction of relative mRNA levels of *ATP6*, *ATP8*, *BMP-15* and *GDF-9* caused by oocyte denudation (Fig. 4), indicating that melatonin significantly improved the quality of oocytes. This moderated reduction may be due to decreased ROS levels in melatonin-treated oocyte, as ROS may degrade the mRNA in oocytes. To our knowledge, this was the first report concerning the effect of melatonin on the gene expression on bovine oocytes.

Melatonin increased the developmental potential of MII oocytes

Many *in vivo* or *in vitro* studies have shown that melatonin could increase the development potential of COCs. When goats (Berlinguer *et al.*, 2009) or sheep (Vázquez *et al.*, 2010) received the melatonin implants, the cleavage and blastocyst rates of COCs after IVF were increased significantly. Manjunatha *et al.* (2009) reported that enrichment of buffalo COCs maturation medium with 10 μM melatonin improved their cleavage and blastocyst rates after IVF.

As removal of the cumulus before IVM caused a precocious exocytosis of cortical granules, leading to zona hardening and reduced penetrability of oocytes by sperm (Ge *et al.*, 2008b), parthenogenetic activation was used to assess the development potential of oocytes in this study. In our experiments, supplementation of DOs IVM medium with 10^{-9} M melatonin increased the blastocyst rate after parthenogenetic activation (24.17 ± 3.54% vs. 35.26 ± 4.87%; $P < 0.05$) and the cell number of parthenogenetic blastocysts (52.74 ± 6.13 vs. 74.92 ± 5.82; $P < 0.05$), similar to results obtained in porcine COCs (Shi *et al.*, 2009). In contrast with the results of Shi *et al.* (2009), 10^{-9} M melatonin did not significantly increase the cleavage rates of oocytes after parthenogenetic activation in this study, similar to the report of Kang *et al.* (2009). Several factors, such as the species (porcine vs. bovine) and

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cell types tested (COCs vs. DOs), may explain these differing results.

Excessive ROS can exacerbate mitochondrial dysfunction (Lin & Beal, 2006), decrease intracellular ATP concentrations, reduce the GSH/GSH disulphide (GSSG) ratios and increase the cytosolic calcium ion concentration, leading to the detrimental effects on oocyte and embryo development (Tarín, 1996). Many studies have indicated that the endogenous antioxidant systems (Gupta *et al.*, 2010) and mitochondrial function (Zhao *et al.*, 2011a,b) could directly influence the developmental potential of oocytes or embryos; therefore, the increased developmental potential of DOs treated with melatonin may be due to the lower level of ROS observed the DOs + MT group, as discussed previously.

Although the development potential of DOs was increased significantly by melatonin, it remained significantly lower than COCs, indicating that the oocyte maturation and quality were also influenced by other cumulus cell-derived factors. The increased developmental potential of the COCs group was probably due to a combination of cumulus cell-derived factors, and were associated with a lower apoptotic rate (Fig. 3) and increased expression of the oocyte quality-markers *ATP6*, *ATP8*, *BMP-15* and *GDF-9* (Fig. 4), compared with the DOs and DOs + MT groups.

Melatonin increased the expression of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* in parthenogenetic blastocysts

To investigate the effect of melatonin on gene expression in parthenogenetic blastocysts, we quantified the expression levels of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3.* As an important factor in regulating bovine blastocysts implantation, embryonic *IFN-τ* expression and secretion level can be utilized as useful tools to evaluate the developmental competence and quality of embryos (Yao *et al.*, 2009). Expressions of *Na^+/K^+-ATPase* and aquaporin play an important role in the formation of tight junctions between trophoblast cells (Kidder & Watson, 2005), and *CTNNBL1* (an important gene in intracellular signalling) has been described as an important marker of compaction and trophectoderm differentiation (Mamo *et al.*, 2011).

The increased expression levels of *IFN-τ*, *Na^+/K^+-ATPase*, *CTNNBL1* and *AQP3* in blastocysts from the DOs+ MT group (Fig. 5) indicated that melatonin may improve the quality of the parthenogenetic blastocysts, which may be a result of the improved oocyte quality of melatonin-treated DOs (Fig. 3 and Table 4). In 2009, Berlinguer *et al.* observed that subcutaneous melatonin implants (18 mg) in goats did not significantly affect the expression levels of β-actin, heat shock protein 90β, cyclin b1, *Na^+/K^+-ATPase*, Type I cadherin or *AQP3* in blastocysts produced by oocyte IVF. The differences between our results and the report of Berlinguer *et al.* (2009) suggested that the effect of melatonin may depend on the treatment method (supplement vs. implant), species (bovine vs. goat) and the method of producing blastocysts (parthenogenetic activation vs. IVF).

Many previous studies have reported that melatonin could regulate different specific gene expression in animals, such as quail (Ubuka *et al.*, 2005), sheep (Johnston *et al.*, 2006) and rat (Park *et al.*, 2007). In our experiment, melatonin was found to increase the quality-marker genes of oocytes and their parthenogenetic blastocysts. It may be that melatonin, having a highly lipophilic nature, can cross the membrane of the target cells and bind to an orphan nuclear receptor to modulate gene expression of certain proteins (Becker-André *et al.*, 1994; Carlberg &Wiesenberg, 1995; Vanecek, 1995; Ubuka *et al.*, 2005).

In conclusion, this study demonstrated that melatonin could improve the nuclear maturation of bovine DOs, increase the expression of oocyte quality-marker genes, decrease ROS levels and reduce the apoptotic rates of MII oocytes in absence of cumulus cells, which contributed to an increased parthenogenetic blastocyst formation rate and quality. These results might enable researchers to more clearly understand the mechanisms by which melatonin could improve the IVM and development potential of oocytes. In future, more information will be obtained when COCs instead of DOs are chosen as the model and IVF instead of parthenogenetic activation is selected to detect the developmental potential of MT-treated oocytes.

Declaration of interest

None

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