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Melatonin reduces apoptotic cells, SOD2 and HSPB1 and improves the in vitro production and quality of bovine blastocysts

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Contents

Effects of adding different concentrations of melatonin (10^{-7}, 10^{-9} and 10^{-11} M) to maturation (Experiment 1; Control, IVM + 10^{-7}, IVM + 10^{-9}, IVM + 10^{-11}) and culture media (Experiment 2; Control, IVC + 10^{-7}, IVC + 10^{-9}, IVC + 10^{-11}) were evaluated on in vitro bovine embryonic development. The optimal concentration of melatonin (10^{-9} M) from Experiments 1–2 was tested in both maturation and/or culture media of Experiment 3 (Control, IVM + 10^{-9}, IVC + 10^{-9}, IVM/IVC + 10^{-9}). In Experiment 1, maturated oocytes from Control and IVM + 10^{-9} treatments showed increased glutathione content, mitochondrial membrane potential and percentage of Grade I blastocysts (40.6% and 43%, respectively). In Experiment 2, an increase in the percentage of Grade I blastocysts was detected in IVC + 10^{-7} (43.5%; 56.7%) and IVC + 10^{-9} (47.4%; 57.4%). Moreover, a lower number and percentage of apoptotic cells in blastocysts were observed in the IVC + 10^{-9} group compared to Control (3.8 ± 0.6; 3.6% *versus* 6.1 ± 0.6; 5.3%). In Experiment 3, the IVC + 10^{-9} treatment increased percentage of Grade I blastocysts with a lower number of apoptotic cells compared to IVM/IVC + 10^{-9} group (52.6%; 3.0 ± 0.5 *versus* 46.0%; 5.4 ± 1.0). The IVC + 10^{-9} treatment also had a higher mRNA expression of antioxidant gene (SOD2) compared to the Control, as well as the heat shock protein (HSPB1) compared to the IVM + 10^{-9}. Reactive oxygen species production was greater in the IVM/IVC + 10^{-9} treatment group. In conclusion, the 10^{-9} M concentration of melatonin and the in vitro production phase in which it is used directly affected embryonic development and quality.

1 | INTRODUCTION

Reactive oxygen species (ROS) are produced during aerobic metabolism and participate in physiological processes that maintain various biological and reproductive functions, including sperm capacitation, fertilization and gestation (Al-Gubory, Fowler, & Garrel, 2010; Burton & Jauniaux, 2011). However, high endogenous ROS concentrations can lead to deleterious effects on cellular functions due to the occurrence of redox imbalance (Takahashi, 2012). Conversely, mammalian cells have highly complex antioxidant systems (enzymatic and non-enzymatic) to protect cells from oxidative damage by "antioxidant" enzymes such as glutathione peroxidase (GSH), catalase and superoxide dismutase (SOD) (Crocomo, Marques Fillho, Landin-Alvarenga, & Bicudo, 2012; Guérin, El Mouatassim, & Ménézo, 2001).

During in vitro culture, oocytes, sperm and embryos are exposed to high oxygen concentration (Kitagawa, Suzuki, Yoneda, & Watanabe, 2004). The absence of the intra-oviductal and intra-uterine environment protection reduces the production of antioxidants and a redox imbalance occurs (Guérin et al., 2001; Kitagawa et al., 2004). Consequently, ROS production increases, and there are deleterious

effects on DNA repair, organization of the mitotic spindle, oocyte maturation and impairs the embryonic development (Agarwal, Said, Bedaiwy, Banerjee, & Alvarez, 2006). Thus, in order to provide better conditions for either in vitro or in vivo embryonic development, it is important to maintain the balance of the intracellular redox state (Takahashi, 2012).

The analysis of oxidation status has been used as a method to estimate the risk of oxidative damage during the culture of gametes and embryos in both animal models and humans (Gomes-Sobrinho et al., 2011). Therefore, oxidative stress detection with direct or indirect measures such as ROS production, amount of intracellular GSH, DNA damage or gene expression could be used to develop and evaluate strategies to prevent or minimize oxidative dysfunction during embryonic development (Kwak et al., 2012; Mukherjee et al., 2014). Furthermore, the inclusion of antioxidants or free radical scavengers to the maturation (Casao et al., 2010; Tian et al., 2014) and culture medium (Wang et al., 2013, 2014) may reduce oxidative stress and improve conditions for embryonic development.

In the set of antioxidant substances, melatonin (*N*-acetyl-5-methoxytryptamine) has been the focus of studies for in vitro embryonic development in humans (Eryilmaz et al., 2011; Khan et al., 2015), mice (Ganji, Nabiuni, & Faraji, 2015; Ren et al., 2015; Wang et al., 2013), sheep (Vázquez, Abecia, Forcada, & Casao, 2010), pig (Li et al., 2015; Shi et al., 2009), buffalo (Manjunatha, Devaraj, Gupta, Ravindra, & Nandi, 2009), rabbit (Mehaisen & Saeed, 2013; Mehaisen et al., 2015) and cattle (Tian et al., 2014; Wang et al., 2014). Melatonin acts directly on ROS, stimulates the activity of endogenous antioxidant enzymes such as SOD, glutathione reductase and catalase and inhibits the activity of pro-oxidant enzymes such as cyclooxygenase (Anisimov et al., 2006; Tan et al., 2015).

Although the effects of melatonin on antioxidant and anti-apoptotic activity during in vitro embryonic (IVP) process are well known (Gomes-Sobrinho et al., 2011; Tamura et al., 2012; Tan et al., 2015), we hypothesized that the effects of melatonin depend on the concentration and phase of the IVP procedure when it is added to the medium. Therefore, the aim of this study was to assess the effects of adding different concentrations of melatonin to the maturation and/or culture medium on in vitro bovine embryonic development. The following endpoints were measured: (i) cleavage and blastocyst rates, (ii) concentrations of glutathione and ROS, (iii) mitochondrial membrane potential, (iv) blastomere apoptosis and (v) the expression of genes related to metabolism, oxidative stress and placentation.

2 | MATERIALS AND METHODS

2.1 | Oocyte selection and in vitro maturation

Bovine ovaries of zebu breeds were collected from local slaughterhouses, transported to the laboratory in a saline solution (0.9%) at 35°C and processed within 4 hr. Cumulus–oocyte complexes (COCs; *n* = 2,767, five replicates per treatment) were aspirated from follicles between 3 and 8 mm in diameter and stored in 15 ml conical polystyrene tubes, in a 36°C water bath for 10 min before decantation.

For each treatment, groups of 30–35 COCs with a homogeneous cytoplasm and several cell layers were selected (Stojkovic et al., 2001) and cultured for in vitro maturation in TCM-199 with Earle's salts and L-glutamine (Gibco®; Invitrogen Co., Grand Island, NY, USA) supplemented with 10% foetal bovine serum (v/v), 0.2 mM pyruvate, 5 mg/ml luteinizing hormone (Lutropin-V®; Bioniche Co., Belleville, ON, Canada), 1 mg/ml follicle-stimulating hormone (Folltropin®; Bioniche Co.), 75 μg/ml amikacin and 1 mM cystamine. The COCs were matured in drops (200 μl) covered with mineral oil (Corning, NY, USA) for 22–24 hr at 38.5°C in 5% CO_2 in air and saturated relative humidity without condensation.

2.2 | In vitro fertilization

After the maturation period, the COCs were washed in fertilization medium TALP-FERT (Tyrode's albumin lactate pyruvate (TALP) supplemented with 6 mg/ml bovine serum albumin (BSA)—fatty acid-free, 0.2 mM pyruvate, 30 μg/ml heparin, 20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine and 75 mg/ml amikacin) and fertilized in drops (200 μl) of the same medium.

For in vitro fertilization (IVF), conventional commercial frozen semen from Nellore bull with known and tested in vitro fertility was used. After thawing (35°C for 30 s), the sperm cells were obtained after treatment with a 45%–90% Percoll gradient as previously described (Parrish, Krogenaes, & Susko-Parrish, 1995). Each drop of COCs was fertilized with a final concentration of 1×10^6 spermatozoa/ml. The sperm cells were co-incubated with oocytes for 18 hr under the same conditions described for maturation.

2.3 | In vitro embryo culture

Presumptive zygotes were denuded partially by repeated pipetting, then washed and transferred into drops (200 μl) of modified synthetic oviduct fluid (Holm, Schimidt, Greve, & Callesen, 1999) supplemented with 2.7 mM myo-inositol, 0.2 mM pyruvate, 2.5% foetal bovine serum (v/v), 5 mg/ml BSA—fatty acid-free and 75 μg/ml amikacin. Embryo culture was performed for 7 days at 38.5°C in 5% CO_2 in air and saturated relative humidity without condensation. After 72 hr of culture, the cleavage rate was assessed and half of the culture medium in each drop was replaced with fresh medium. Structures were considered cleaved when the cellular division with at least 2–4 cells was present. The morphological quality of the embryos was measured according to International Embryo Transfer Society (IETS) guidelines. According to IETS, the quality of the embryos is classified in Grade 1 to 4 (1—excellent/good, 2—regular, 3—poor, 4—unviable). Embryos are graded regarding the number of physical characteristics including shape, colour and density of cytoplasm or inner cell mass, number and compactness of cells, area of perivitelline space, number of extruded or degenerate cells and frequency and size of cytoplasmic vacuoles. The per cent blastocyst at day 7 (D7) was evaluated, where day 0 (D0) is the day of IVF.

2.4 | Experimental design

2.4.1 | Experiment 1—Effect of melatonin added to maturation medium on in vitro bovine embryonic development

After selection, COCs were placed into drops of maturation medium without (Control) or supplemented with different concentrations (10^{-7}, 10^{-9} and 10^{-11} M) of melatonin (Sigma Co., St. Louis, MO, USA) represented by the treatments: IVM + 10^{-7}, IVM + 10^{-9} and IVM + 10^{-11}, respectively. MMP, ROS and GSH were measured in oocytes after 24 hr of maturation. Also, cleavage, blastocyst formation and quality were assessed.

2.4.2 | Experiment 2—Effect of melatonin added to culture medium on in vitro bovine embryonic development

After maturation and fertilization, presumptive zygotes were transferred into drops of culture medium without (Control) or supplemented with different concentrations of melatonin (10^{-7}, 10^{-9} and 10^{-11} M) represented by the treatments IVC + 10^{-7}, IVC + 10^{-9} and IVC + 10^{-11}, respectively. Cleavage, blastocyst formation and quality were evaluated. Moreover, total cell number and number of apoptotic cells in the blastocysts were measured.

2.4.3 | Experiment 3—Effect of melatonin added to maturation and culture medium on in vitro bovine embryonic development

After evaluation of embryonic development and analysis of GSH, MMP and apoptosis, the better results from Experiment 1 (IVM + 10^{-9}) and Experiment 2 (IVC + 10^{-9}) were associated with the maturation and culture medium (IVM/IVC + 10^{-9}) and compared to the Control group. Cleavage, blastocyst formation and quality, GSH, ROS, total cell number and cellular apoptosis in blastocysts were evaluated. In addition, embryo viability was analysed by expression of five genes related to metabolism (SLC2A1, SLC2A3), oxidative stress (HSPB1, SOD2) and placentation (KRT8).

2.5 | Intracellular measurement of reactive oxygen species (ROS) and glutathione (GSH)

Intracellular content of ROS and GSH was measured in denuded oocytes matured in vitro (Experiment 1) and in D7 blastocysts (Experiment 3), as described previously (Park & Yu, 2013) with some modifications. Briefly, to evaluate ROS and GSH, 10 mM 6-carboxy -2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes®, Eugene, OR) and 10 mM 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker™ Blue CMF_2HC, Molecular Probes®, Eugene, OR) were used, respectively. Oocytes and embryos were washed in 0.1% polyvinyl alcohol in phosphate-buffered saline (PBS-PVA) and incubated concurrently with both probes at 38.5°C in the dark for 30 min. After incubation, the oocytes and embryos were washed with PBS-PVA, placed onto glass slides with ProLong®Gold (Molecular Probes, Eugene, OR) and cover-slipped. Then, the slides were brought to the epifluorescence microscope (Olympus BX43, Tokyo, Japan). First, under the 460 nm wavelength filter for observation and image capture of ROS. Immediately afterwards, the same procedure was performed for GSH with a 370-nm wavelength filter.

Images were obtained using a camera (Olympus SC30, Tokyo, Japan) coupled to the epifluorescence microscope and saved in a TIFF format. The fluorescence intensities of the oocytes and embryos were analysed individually using Image J software (version 1.46; National Institutes of Health, Bethesda, MD) and normalized to the Control group. The relative fluorescence intensity was considered directly proportional to ROS and GSH concentrations.

2.6 | Measurement of mitochondrial membrane potential (MMP)

In Experiment 1, the mitochondrial membrane potential of the oocytes was measured after 24 hr of in vitro maturation using a fluorescent indicator for mitochondrial activity (thiol-chloromethyl), according to the method previously described (Santos, Sato, Lucia, & Iwata, 2013). Oocytes were denuded, washed in PBS-PVA and kept at 38.5°C for 30 min in PBS-PVA with 0.5 μM $C_{24}H_{24}Cl_2N_2O$ (MitoTracker Orange CMTMRos®; Life Technologies Corporation, Carlsbad, CA, USA). Next, oocytes were washed in PBS-PVA, fixed in 3.7% paraformaldehyde for 24 hr at 4°C protected from light. Afterwards, they were washed in PBS-PVA, placed onto glass plates with ProLong®Gold (Molecular Probes, Eugene, OR), cover-slipped and examined under an epifluorescence microscope (Olympus BX43, Tokyo, Japan) with a 550-nm wavelength filter. Images were obtained, and the light intensity emitted by each oocyte was analysed as previously described for ROS and GSH.

2.7 | Analysis of apoptotic cells

Blastomere apoptosis was detected using the TUNEL technique (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling), with a commercial In Situ Cell Death Detection kit (Roche, Mannheim, Germany). Experimental groups were prepared according to manufacturer's instructions: treatments, positive Control and negative Control. Blastocysts (D7) were washed in 0.1% polyvinylpyrrolidone in phosphate-buffered saline (PBS-PVP) and then fixed in 3.7% paraformaldehyde diluted in PBS-PVP for 1 hr at room temperature. Shortly afterwards, they were incubated in a membrane permeabilization solution (PBS containing 0.5% Triton X-100 and sodium citrate) for 1 hr at 4°C. Next, the positive Control group was incubated in a DNAse solution for 1 hr in a humid chamber at 37°C, while the other groups remained in PBS-PVP on a hot plate at 37°C. Afterwards, the blastocysts were incubated for the TUNEL reaction (treated and positive groups in the enzyme marker solution [1:9]; negative group in the marker solution only) for 1 hr in a humid chamber at 37°C with protection from light. Next, all groups were

TABLE 1 Specific primers used to amplify gene fragments for quantitative real-time PCR analysis

Gene	Primer sequence	Size (bp)	Primer concentration (nM)	GenBank Access/ Reference No.
GAPDH	F: GGC GTG AAC CAC GAG AAG TAT AA R: CCC TCC ACG ATG CCA AAG T	118	300	NM_001034034.2
SLC2A1	F: CAG GAG ATG AAG GAG GAG AGC R: CAC AAA TAG CGA CAC GAC AGT	258	250	BT029806
SLC2A3	F: ACT CTT CAC CTG ATT GGC CTT GGA R: GGC CAA TTT CAA AGA AGG CCA CGA	145	300	NM_174603.3
KRT8	F: TGT GAA GAA GAT TGA GAC CCG CGA R: AAA CCT CAG GTC TCC TGT GCA GAT	160	300	X12877 (El-Sayed et al., 2006)
SOD2	F: TTG CTG GAA GCC ATC AAA CGT GAC R: AAT CTG TAA GCG TCC CTG CTC CTT	135	300	NM_201527
HSPB1	F: CTG GAC GTC AAC CAC TTC R: GGA CAG AGA GGA GGA GAC	180	250	NM_001025569.1

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; SLC2A1 = *Bos taurus* solute carrier family 2 (facilitated glucose transporter), member 1; SLC2A3 = *Bos taurus* solute carrier family 2 (facilitated glucose transporter), member 3; KRT8 = keratin 8; SOD2 = *Bos taurus* superoxide dismutase 2; HSPB1 = *Bos taurus* heat shock 27 kDa protein 1. F: forward primer; R: reverse primer.

TABLE 2 In vitro embryonic development and quality maturated with different concentrations of melatonin (Experiment 1)

Treatment	Cleavage (%)	Blastocyst (%)	Blastocyst quality (%)	
			Grade I	Grade II
Control[†] (*n* = 254)	75.6 (192/254)[b]	39.8 (101/254)[a]	40.6 (41/101)[ab]	38.6 (39/101)[b]
IVM + 10^{-7} (*n* = 260)	74.6 (194/260)[b]	36.2 (94/260)[a]	27.7 (26/94)[b]	36.2 (34/94)[b]
IVM + 10^{-9} (*n* = 253)	88.9 (225/253)[a]	42.3 (107/253)[a]	43.0 (46/107)[a]	34.6 (37/107)[b]
IVM + 10^{-11} (*n* = 261)	83.1 (217/261)[a]	44.4 (116/261)[a]	10.3 (12/116)[c]	69.0 (80/116)[a]

[†]Number of oocytes subjected to in vitro maturation. Control: medium without melatonin; maturation medium supplemented with 10^{-7} (IVM + 10^{-7}), 10^{-9} (IVM + 10^{-9}) and 10^{-11} M (IVM + 10^{-11}) of melatonin. [a,b,c]Within a column, values without a common superscript differed among treatments ($p < .05$).

incubated in a Hoechst 33342 solution (Invitrogen Corp., Carlsbad, CA) for 10 min, protected from light. ProLong®Gold (Molecular Probes, Eugene, OR) was used to mount the slides and coverslips for observation with an epifluorescence microscope (Olympus BX43, Tokyo, Japan). Between each stage, the groups were washed with three drops of PBS-PVP, remaining for 2 min in the last drop. Nuclei of the TUNEL-positive cells—that is, those that contain fragmented DNA—were visualized under green fluorescence at a wavelength of 450 nm. All nuclei were visualized under blue fluorescence at 365 nm. Nuclei were counted to obtain the total number of cells (TNC, blue) and the number of apoptotic cells (NAC, green) in embryos from each group.

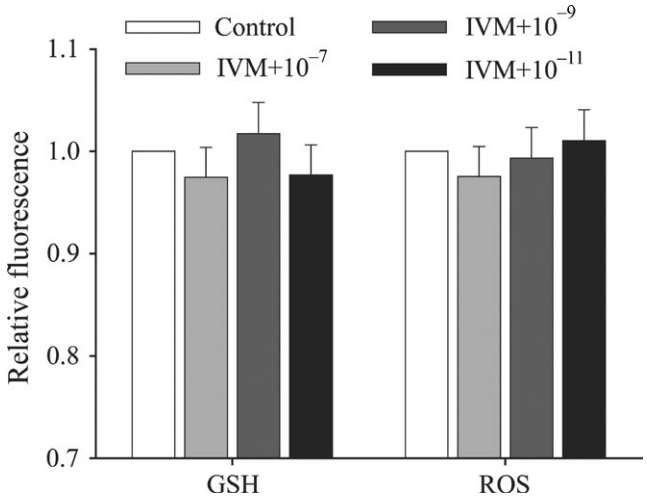

FIGURE 1 Intracellular levels of glutathione (GSH) and reactive oxygen species (ROS) in oocytes (n = 152) matured in vitro with different concentrations of melatonin. Control: medium without melatonin; maturation medium supplemented with 10^{-7} (IVM + 10^{-7}), 10^{-9} (IVM + 10^{-9}) and 10^{-11} M (IVM + 10^{-11}) of melatonin. Fluorescence intensity in the Control group was defined as 1.0. [a,b,c]Within the same parameter evaluated, values without a common letter differed (p < .001)

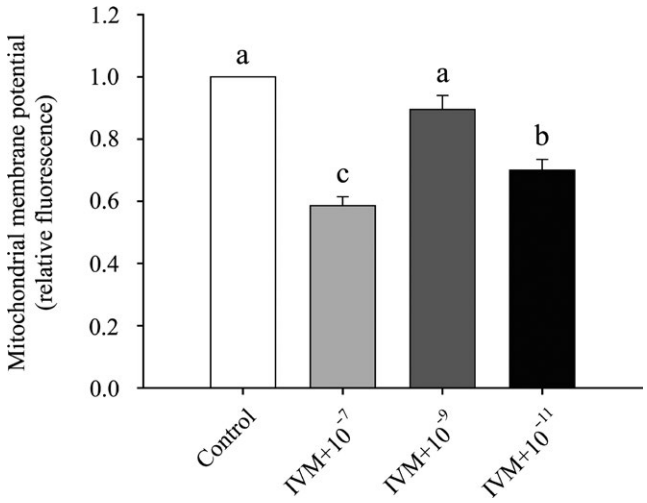

FIGURE 2 Mitochondrial membrane potential (MMP) in oocytes (n = 140) matured in vitro with different concentrations of melatonin. Control: medium without melatonin; maturation medium supplemented with 10^{-7} (IVM + 10^{-7}), 10^{-9} (IVM + 10^{-9}) and 10^{-11} M (IVM + 10^{-11}) of melatonin. Fluorescence intensity in the Control group was defined as 1.0. [a,b,c]Values without a common letter differed (p < .001)

2.8 | Sample preparation, RNA extraction and cDNA production

Blastocysts (D7) were washed in PBS without calcium and magnesium and transferred into 2 μl of PBS plus 4 μl of RNAlater (Life Technologies Corporation, Carlsbad, CA, USA) into DNAse/RNAse-free polypropylene microtubes. After incubation at 4°C for 12 hr, they were stored at −20°C until RNA extraction.

Embryo viability was evaluated by the expression of five genes related to metabolism (SLC2A1, SLC2A3), oxidative stress (HSPB1, SOD2) and placentation (KRT8), using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Three pools of 15 blastocysts were used for each treatment.

Total RNA was isolated using an RNeasy Plus Micro Kit (Qiagen®, Hilden, Germany) according to manufacturer's instructions. The total amount of isolated RNA was used to synthesize cDNA using a commercial First-Strand cDNA Synthesis kit (Invitrogen)—SuperScript® III (200 U/μl) and oligo-dT primer (0.5 μg/μl) to a final volume of 40 μl. Reactions were performed at 65°C for 5 min and 50°C for 50 min, followed by inactivation of the enzyme at 85°C for 5 min. The qPCR analysis was performed using the Fast Sybr Green Master Mix (Applied Biosystems). Reactions were optimized to promote the maximum amplification efficiency for each gene (76%–110%), calculated using the standard relative curves in the software program 7500 2.0.3 (Applied Biosystems). Each sample was analysed in triplicate, and the specificity of each PCR product was determined through analysis of the melting curve and amplicon size on an agarose gel. Reactions were performed in a final volume of 25 μl using a cDNA amount equivalent to 0.5 embryos. The conditions for the PCR reaction were as follows: 95°C for 5 min, 50 cycles at 95°C for 10 s and 60°C for 30 s. The name, primer sequence and concentration, amplicon size and annealing temperature for each gene are listed in Table 1.

Expression levels of the three constituent genes—glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and peptidyl-prolyl isomerase A (PPIA)—were analysed using geNorm software, which determined that GAPDH was the most stable gene. GAPDH was therefore used as a reference to normalize the data. The relative expression of each gene was calculated using the $\Delta\Delta C_t$ method with efficiency correction (Pfaffl, 2001).

2.9 | Statistical analyses

All statistical analyses were performed using R software version 3.0.2 (R Core Team, 2014). Data that were not normally distributed by the Shapiro–Wilk test were submitted to natural log transformation. Reactive oxygen species, glutathione, mitochondrial membrane potential and gene expression were analysed among treatments by Kruskal–Wallis test. Total cell number, number of apoptotic cells and mitochondrial membrane potential were compared among treatments by Tukey test. The cleavage and blastocyst rates and blastocyst quality were compared among treatments using the chi-square test. Data are presented as mean (± standard deviation) and percentage. Results were considered significantly different at p < .05 (two-sided).

3 | RESULTS

3.1 | Experiment 1

Addition of melatonin to the maturation medium at concentrations of 10^{-9} (IVM + 10^{-9}) and 10^{-11} M (IVM + 10^{-11}) increased (p < .05) the

TABLE 3 Effects of different concentrations of melatonin added to culture medium on embryonic development and quality (Experiment 2)

Treatment	Cleavage (%)	Blastocyst (%)	Blastocyst quality (%)	
			Grade I	Grade II
Control† (*n* = 221)	79.6 (176/221)[ab]	37.1 (82/221)[bc]	39.9 (32/82)[b]	34.1 (28/82)[a]
IVC + 10^{-7} (*n* = 223)	75.8 (169/223)[bc]	43.5 (97/223)[ab]	56.7 (55/97)[ac]	28.9 (28/97)[a]
IVC + 10^{-9} (*n* = 228)	84.2 (192/228)[a]	47.4 (108/228)[a]	57.4 (62/108)[ac]	27.8 (30/108)[a]
IVC + 10^{-11} (*n* = 228)	70.6 (161/228)[c]	31.1 (71/228)[c]	50.7 (36/71)[bc]	35.2 (25/71)[a]

†Number of oocytes subjected to in vitro maturation. Control: medium without melatonin; culture medium supplemented with 10^{-7} (IVC + 10^{-7}), 10^{-9} (IVC + 10^{-9}) and 10^{-11} M (IVC + 10^{-11}) of melatonin. [a,b,c]Within a column, values without a common superscript differed among treatments ($p < .05$).

TABLE 4 Effects of different concentrations of melatonin added to culture medium on the total cell number and apoptotic cells (mean ± standard deviation) in blastocysts produced in vitro (Experiment 2)

Treatments	Total cell number	Number of apoptotic cells (%)
Control† (*n* = 20)	115.9 ± 4.0[a]	6.1 ± 0.6 (5.3)[b]
IVC + 10^{-7} (*n* = 20)	115.1 ± 3.8[a]	6.3 ± 0.5 (5.5)[b]
IVC + 10^{-9} (*n* = 20)	103.3 ± 4.1[a]	3.8 ± 0.6 (3.6)[a]
IVC + 10^{-11} (*n* = 20)	111.4 ± 4.0[a]	5.1 ± 0.6 (4.6)[ab]

†Number of blastocysts evaluated. Control: medium without melatonin; culture medium supplemented with 10^{-7} (IVC + 10^{-7}), 10^{-9} (IVC + 10^{-9}) and 10^{-11} M (IVC + 10^{-11}) of melatonin. [a,b]Within a column, values without a common superscript differed among treatments ($p < .05$).

rate of embryonic cleavage, but did not improve the blastocyst formation (Table 2). Also, the percentage of blastocysts Grade I was lower ($p < .05$) in IVM + 10^{-7} and IVM + 10^{-11} treatments in comparison with the IVM + 10^{-9}. Percentage of blastocysts Grade II was higher for the IVM + 10^{-11}.

The intracellular levels of GSH and ROS in oocytes after in vitro maturation are presented in Figure 1. Oocytes in the IVM + 10^{-9} treatment showed an increase in GSH ($p < .001$), while those in the IVM + 10^{-7} treatment showed a lower amount of ROS ($p < .001$). In addition, a lower amount of MMP was seen in IVM + 10^{-7} and IVM + 10^{-11} groups compared to Control and IVM + 10^{-9} treatments ($p < .001$; Figure 2).

3.2 | Experiment 2

The embryonic development and quality after in vitro culture in medium supplemented with different concentrations of melatonin are shown (Table 3). Greater percentage ($p < .05$) of cleavage was detected in the IVC + 10^{-9} in comparison with IVC + 10^{-7} and IVC + 10^{-11}. Also, IVC + 10^{-9} treatment had a higher ($p < .05$) percentage of blastocysts compared to the Control group. Furthermore, a higher ($p < .05$) percentage of Grade I blastocysts occurred in the in the IVC + 10^{-7} and IVC + 10^{-9} treatments compared with Control group.

TABLE 5 Percentage of cleavage, blastocyst formation and quality after the addition of melatonin at 10^{-9} M in different phases of in vitro production (Experiment 3)

Treatment	Cleavage (%)	Blastocyst (%)	Blastocyst quality (%)	
			Grade I	Grade II
Control† (*n* = 216)	83.3 (180/216)[a]	41.7 (90/216)[b]	35.6 (32/90)[b]	34.4 (31/90)[a]
IVM + 10^{-9} (*n* = 201)	86.6 (174/201)[a]	47.3 (95/201)[ab]	44.2 (42/95)[b]	31.6 (30/95)[a]
IVC + 10^{-9} (*n* = 216)	82.9 (179/216)[a]	52.8 (114/216)[a]	52.6 (60/114)[a]	28.9 (33/114)[a]
IVM/IVC + 10^{-9} (*n* = 206)	79.6 (164/206)[a]	42.2 (87/206)[b]	46.0 (40/87)[b]	36.8 (32/87)[a]

†Number of oocytes subjected to in vitro maturation. Control: medium without melatonin; IVM + 10^{-9}: maturation medium supplemented with 10^{-9} M of melatonin; IVC + 10^{-9}: culture medium supplemented with 10^{-9} M of melatonin; IVM/IVC + 10^{-9}: maturation and culture medium supplemented with 10^{-9} M of melatonin. [a,b]Within a column, values without a common superscript differed among treatments ($p < .05$).

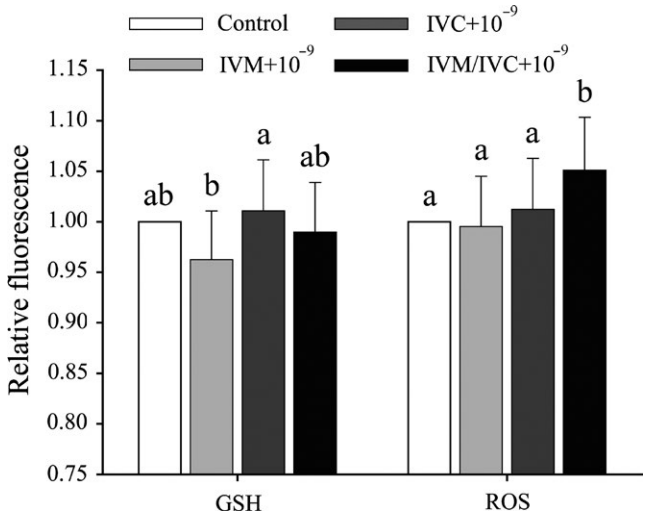

FIGURE 3 Intracellular levels of glutathione (GSH) and reactive oxygen species (ROS) in blastocysts (n = 60) after the addition of melatonin at 10^{-9} M in different phases of in vitro production. Control: medium without melatonin; IVM + 10^{-9}: maturation medium supplemented with 10^{-9} M of melatonin; IVC + 10^{-9}: culture medium supplemented with 10^{-9} M of melatonin; IVM/IVC + 10^{-9}: maturation and culture medium supplemented with 10^{-9} M of melatonin. Fluorescence intensity in the Control group was defined as 1.0. [a,b]Within the same parameter evaluated, values without a common letter differed (p < .001)

TABLE 6 Total cell number and apoptotic cells (mean ± standard deviation) in blastocysts after the addition of melatonin at 10^{-9} M in different phases of in vitro production (Experiment 3)

Treatment	Total cell number	Number of apoptotic cells (%)
Control † (n = 16)	135.0 ± 7.2[a]	6.5 ± 0.6 (4.8)[b]
IVM + 10^{-9} (n = 15)	130.6 ± 6.7[a]	5.3 ± 0.6 (4.0)[b]
IVC + 10^{-9} (n = 18)	116.6 ± 5.0[a]	3.0 ± 0.5 (2.5)[a]
IVM/IVC + 10^{-9} (n = 15)	130.2 ± 7.3[a]	5.4 ± 1.0 (4.1)[b]

†Number of blastocysts evaluated. Control: medium without melatonin; IVM + 10^{-9}: maturation medium supplemented with 10^{-9} M of melatonin; IVC + 10^{-9}: culture medium supplemented with 10^{-9} M of melatonin; IVM/IVC + 10^{-9}: maturation and culture medium supplemented with 10^{-9} M of melatonin. [a,b]Within a column, values without a common superscript differed among treatments (p < .05).

TUNEL analysis of blastocysts (Table 4) showed a lower (p < .05) number of apoptotic cells with melatonin concentration of 10^{-9} M in the culture medium compared to the Control group.

3.3 | Experiment 3

The effects of melatonin (10^{-9} M) added to maturation and/or culture medium on in vitro bovine embryonic development are shown (Table 5). A higher blastocyst rate (p < .05) was observed in IVC + 10^{-9} treatment compared to the Control and IVM/IVC + 10^{-9} groups. Additionally, the IVC + 10^{-9} had a greater (p < .05) percentage of blastocysts Grade I.

The intracellular levels of GSH were lower (p < .05) in blastocysts from the IVM + 10^{-9} treatment compared to the IVC + 10^{-9} group (Figure 3). Moreover, ROS production was higher (p < .05) in the group supplemented simultaneously in the maturation and culture medium (IVM/IVC + 10^{-9}).

TUNEL analysis revealed a lower (p < .05) number of apoptotic cells in blastocysts produced in the IVC + 10^{-9} treatment (Table 6). Furthermore, the relative abundance of SOD2 mRNA was higher in the IVC + 10^{-9} treatment compared to the Control, as well as the heat shock protein HSPB1 compared to the IVM + 10^{-9} (p < .05; Figure 4). The expression of genes related to cell metabolism (SLC2A1 and SLC2A3) and placentation (KRT8) was similar (p > .05) between treatments.

4 | DISCUSSION

Melatonin is an antioxidant molecule and can protect gametes and embryos, and we tested whether supplementing media related to the different steps during the in vitro bovine embryo production process with different concentrations of melatonin would improve the cleavage and/or the embryo quality. We showed that the addition of melatonin in the IVM or in both (IVM and IVC) consecutively is not necessary. IVC medium supplemented with 10^{-9} M of melatonin improves embryos quality and increases the number of D7 blastocysts. Additionally, an over-expression of superoxide dismutase (SOD2) compared to the Control and HSPB1 compared to the IVM + 10^{-9}, greater intracellular content of glutathione, and a reduction in apoptosis were observed indicative that this concentration of melatonin utilized and the in vitro production phase under the conditions described directly affected embryonic development.

In the Experiment 1, addition of melatonin to the maturation medium at concentrations of 10^{-9} (IVM + 10^{-9}) and 10^{-11} M (IVM + 10^{-11}) increased cleavage rate, but did not improve blastocyst production. Also, matured oocytes of the IVM + 10^{-9} treatment showed a higher percentage of Grade I blastocysts, greater levels of MMP and GSH and lower ROS production compared with the IVM + 10^{-11} group. Maturation of bovine oocytes was improved at concentrations of 10^{-7} and 10^{-9} M of melatonin, and an upregulated expression of genes associated both with oocyte maturation (GDF9, MARF1, DNMT1o) and expansion of cumulus cells (PTX3, HAS1/2) (Tian et al., 2014). Also, studies (Takeo et al., 2014; Van-Blerkom, Davis, Mathwig, & Alexander, 2002) showed a positive relationship between MMP and matured oocytes. Moreover, high concentrations of GSH were related to increased efficiency in maturation until the time of blastocyst formation (Luberda, 2005). It is known that ROS can cause mitochondrial damage in oocytes (Ge et al., 2012), and the interaction of melatonin with ROS is a prolonged process that involves many of its metabolites, making melatonin highly effective at protecting cells against oxidative stress (Park & Yu, 2013; Tamura et al., 2008; Van-Blerkom et al., 2002).

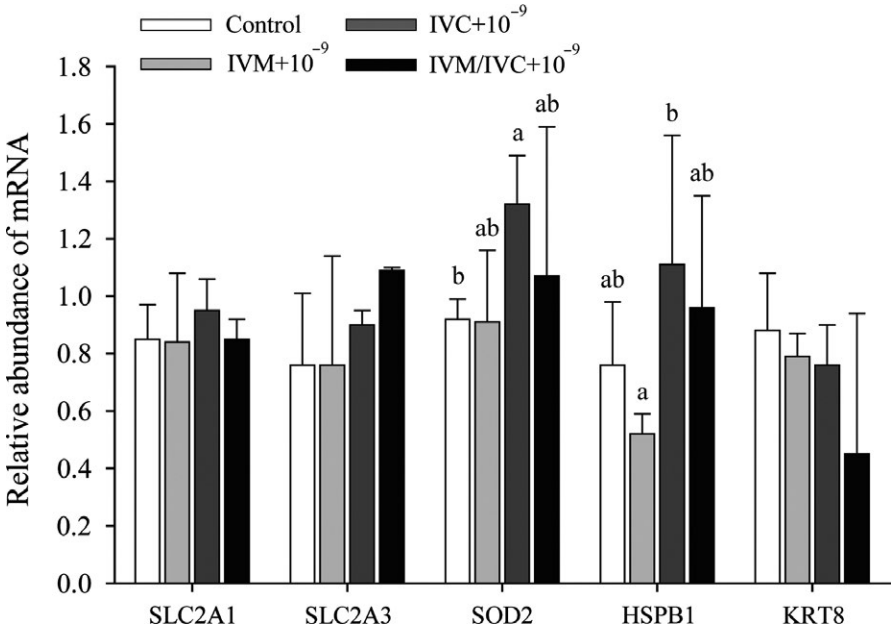

FIGURE 4 Relative mRNA abundance (mean ± standard deviation) of genes related to cell metabolism (SLC2A1, SLC2A3), oxidative stress (SOD2, HSPB1) and placentation (KRT8) in blastocysts after the addition of melatonin at 10^{-9} M in different phases of in vitro production. Control: medium without melatonin; IVM + 10^{-9}: maturation medium supplemented with 10^{-9} M of melatonin; IVC + 10^{-9}: culture medium supplemented with 10^{-9} M of melatonin; IVM/IVC + 10^{-9}: maturation and culture medium supplemented with 10^{-9} M of melatonin. [a,b]Indicate differences among treatments within the same gene evaluated ($p < .05$)

In Experiment 2, IVC + 10^{-9} treatment of culture media increased the percentage of high-quality blastocysts with a decreased number of apoptotic cells compared to the Control group. Furthermore, melatonin at a concentration of 10^{-11} M (IVC + 10^{-11}) had a negative effect on embryo development compared to the IVC + 10^{-9} group. Despite melatonin improvements on in vitro oocyte maturation and embryonic development, melatonin beneficial effects appear to be species-specific (ovine (Abecia, Forcada, & Zúñiga, 2002), rabbit (Mehaisen et al., 2015), porcine (Rodriguez-Osorio, Kim, Wang, Kaya, & Memili, 2007) and mice (Tan et al., 2015)) and concentration-dependent, regardless of the therapeutic responses evaluated (e.g. antitumour activity (Zou et al., 2015), antioxidant protection (Ginther et al., 2012), enhances immune function (Srinivasan et al., 2005), sleep (Dawson & Encel, 1993) and reproduction (Reiter et al., 2009)). Additionally, the melatonin as an antioxidant and free radical scavenger reduces the oxidative damage and dysfunction of oocytes during maturation (Casao et al., 2010; Tian et al., 2014) and embryo culture (Wang et al., 2013, 2014).

Herein, the number of apoptotic cells in blastocysts produced in vitro was used as an indicator of embryo quality (Experiments 2 and 3). Although apoptosis during pre-implantation development of the embryo plays a fundamental role in eliminating defective cells, it can also induce a greater loss of healthy cells, depending of culture conditions (Byrne, Southgate, Brison, & Leese, 1999; Hao et al., 2003). Unlike from this study, recent reports (Sampaio, Conceição, Miranda, Sampaio, & Ohashi, 2012; Wang et al., 2014) indicated that melatonin improves the mean cell number per blastocyst and, consequently, more cells undergoing continual mitosis that contributes to increase the potential development of the embryo (Tian et al., 2014). Moreover, there is a strong relationship of maternal melatonin with pregnancy and normal foetal development (Voiculescu, Zygouropoulos, Zahiu, & Zagrean, 2014). The mechanisms involve the melatonin's ability to downregulate the expression of pro-apoptotic genes (p53, Bax and caspase-3) while upregulating the expression of the antioxidant enzyme (SOD and Gpx4) and the anti-apoptotic gene Bcl-2. These changes lead a decrease in ROS production and cellular apoptosis during embryo development and consequently improve the quality of the cultured embryos (Wang et al., 2013, 2014).

In Experiment 3, the effects of an optimal melatonin concentration (10^{-9} M; Experiments 1 and 2) added to maturation and/or culture medium were evaluated. The addition of melatonin to both maturation and culture medium (IVM/IVC + 10^{-9}) resulted in a lower blastocyst rate and higher number of apoptotic cells than the group supplemented with melatonin (IVC + 10^{-9}) during only the culture period. The beneficial effects of melatonin exhibit a concentration dependence (Tian et al., 2014). In development, melatonin acts by MT1 and MT2 membrane receptors that are expressed in oocytes, cumulus cells and granulosa cells (El-Raey et al., 2011; Tian et al., 2014), and its most effective concentration tested in vitro was related to presence of the MT1 melatonin receptor (Sampaio et al., 2012). Interestingly, the same report showed that only the MT1 receptor is present at the blastocyst stage indicative that melatonin has effects on oocyte maturation and embryo development via membrane receptor activation. Furthermore, most of the MT1 receptor appears in response to low concentrations of melatonin, but the response mediated by MT2 occurs at higher concentrations. Thus, some receptors may be activated during maturation but do not answer to the stimulation during the culture. At higher melatonin concentrations, a residual impact of melatonin by the MT1 receptor is seeming (Jin et al., 2003). This difference in the relative contribution of the receptor subtypes is likely not due to differences in the affinity of the receptors, but may be from differences in the levels of receptor expression or by differences in coupling to second messenger pathways (Jin et al., 2003).

We propose three hypotheses to explain the ROS increase in the IVM/IVC + 10^{-9} group: (i) mitochondrial dysfunction due to a failure in the methylation of DNA cytosine (Su et al., 2015); (ii) unavailability of

membrane receptors for melatonin during culture, as receptors were occupied during maturation and or downregulated during culture and thus cease to activate important pathways (Jin et al., 2003) making it difficult to maintain redox balance; and (iii) receptor damage by ROS during embryonic development, thus minimizing the action of melatonin. The receptor damage by ROS has been observed in mice embryonic development, where melatonin modulates the sensitivity of cones, the photoreceptor cells in the retina, through activation of the MT1 and MT2 receptors (Baba et al., 2014).

Cells are also protected by an enzymatic system that includes superoxide dismutase (SOD) in addition to protection from ROS-induced oxidative damage by non-enzymatic agents (El Mouatassim, Guérin, & Ménézo, 2000). This system transforms the superoxide radical into oxygen and hydrogen peroxide (Roy, Gauvreau, & Bilodeau, 2008). Melatonin works via this system by scavenging free radicals, directly eliminating ROS (Galano, Tan, & Reiter, 2013; Zhang & Zhang, 2014), increasing the expression of genes that encode antioxidant enzymes such as SOD and GSH (Gao et al., 2012) and inhibiting the action of pro-oxidative enzymes to reduce cellular oxidative stress (Zhang & Zhang, 2014).

Much of the cellular changes leading to thermotolerance involve genes activation of heat shock proteins as HSPB1, HSP1A1 and HSPA5 (Lepock, 2005). Under thermal stress conditions, HSPB1 acts synergistically with HSPA1 and this action is critical for preventing cell apoptosis (Showell and Conlon, 2007).

In summary, this study demonstrates the important role of melatonin during the culture phase of IVP in reducing the cellular oxidative stress and protecting blastomeres from cellular death by apoptosis. Melatonin added to culture medium at a concentration of 10^{-9} M proved to be effective at improving the production and quality of bovine blastocysts in vitro, without the need to supplement the maturation medium sequentially in association with the culture medium. These data provide additional strategies for future studies focused on optimization of IVP, cryopreservation of embryos, as well as the potential benefits of adding melatonin to the re-culture medium post-thawing of cryopreserved embryos.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

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