Theriogenology xxx (2016) 1–10

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effects of melatonin during IVM in defined medium on oocyte meiosis, oxidative stress, and subsequent embryo development

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ARTICLE INFO

Article history:
Received 9 December 2015
Received in revised form 2 May 2016
Accepted 20 May 2016

Keywords:
In vitro fertilization
Gene expression
Nuclear fragmentation
Superoxide dismutase
Bovine

ABSTRACT

Melatonin may have beneficial effects when used in oocyte maturation and embryo development culture. The effect of melatonin during IVM on meiosis resumption and progression in bovine oocytes and on expression of antioxidant enzymes, nuclear fragmentation and free radicals, as well as on embryo development were assessed. Cumulus-oocyte complexes were matured *in vitro* with melatonin (10^{-9} and 10^{-6} M), FSH (positive control), or without hormones (negative control) in defined medium. Maturation rates were evaluated at 6, 12, 18, and 24 hours. Transcripts for antioxidant enzymes (*CuZnSOD*, *MnSOD*, and glutathione peroxidase 4 (*GPX4*)) in oocytes and cumulus cells, nuclear fragmentation in cumulus cells (TUNEL) and reactive oxygen species levels in oocytes (carboxy-H_2 difluorofluorescein diacetate) were determined at 24 hours IVM. Effect of treatments on embryo development was determined after *in vitro* fertilization and culture. At 12 hours, meiosis resumption rates in FSH and melatonin-treated groups were similar (69.6%–81.8%, $P > 0.05$). At 24 hours, most oocytes were in metaphase II, with FSH showing highest rates (90.0%, $P < 0.05$) compared with the other groups (51.6%–69.1%, $P > 0.05$). In cumulus cells, *MnSOD* expression was higher in FSH group ($P < 0.05$) whereas *Cu,ZnSOD* transcripts were more abundant in melatonin group (10^{-6}M; $P < 0.05$). Nuclear fragmentation in cumulus cells was highest in controls (37.4%/10,000 cells; $P < 0.05$) and lower in FSH and 10^{-6}M melatonin (29.4% and 25.6%/10,000 cells, respectively). Reactive oxygen species levels were lower in oocytes matured with 10^{-6}M melatonin than in control and FSH groups ($P < 0.05$). Embryo development from oocytes matured only with melatonin was similar to those matured in complete medium ($P > 0.05$). In conclusion, although melatonin during IVM in a defined medium does not stimulate nuclear maturation progression it does stimulate meiosis resumption and such treated oocytes support subsequent embryo development. Melatonin also shows cytoprotective effects on cumulus-oocyte complexes.

1. Introduction

Despite extensive dedication to research and to advances in the *in vitro* production (IVP) of bovine embryos,

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0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.05.026

outcomes are still far from desirable when compared with embryos produced *in vivo*. The developmental competence of oocytes matured *in vitro* is still lower than that of those matured *in vivo* [1].

Lonergan et al. [2] and Rizos et al. [1] raised evidence that embryo development rates are related to the intrinsic quality of oocytes, whereas the quality of embryos is mainly because of IVC conditions. Therefore, IVM culture conditions can exert relevant impact on the production of bovine embryos [3], including effects on nuclear maturation and cleavage and blastocyst rates [4].

Melatonin (N-acetyl-5-metoxy tryptamine) is an indolamine produced from the amino acid tryptophan, not only in the pineal gland but also in others parts of the organism such as retina, extra-orbital lacrimal gland, gastrointestinal tract, skin, and ovary [5–7]. This hormone is well-known for its functions in controlling the circadian rhythm and reproductive seasonality [8], but it also plays an important role in various physiological functions because of its immunomodulatory properties and cytoprotective effects [9,10]. As an antioxidant, it is able to mobilize DNA repair mechanisms [11], directly regulate the action of several antioxidant enzymes, oxidative metabolism, and electron transport [12].

Melatonin has been shown to influence female reproduction and ovarian function [13,14]. In humans, melatonin was found to reduce intrafollicular oxidative stress and improve fertilization rates after oral treatment [15]. Female goats treated with melatonin implants showed increased follicular waves and improved oocyte competence [16]. Besides, the indolamine has been detected in the follicular fluid of humans [17], pigs [18], and, more recently, also in cattle [19], and its receptors were localized in oocytes and cumulus cells [20], suggesting its importance for oocyte development.

Studies *in vitro* also have shown beneficial effects of its use during oocyte maturation and embryo culture *in vitro*, although the results are sometimes contradictory. Melatonin supplementation during IVM has been shown to have no effect [21], to decrease [22] or to improve nuclear maturation [20] and to have no effect [21,23] or to improve subsequent embryo development [24].

Most of these studies have used concomitant addition of serum and/or hormones during IVM, which could be related to the variable results. Therefore, the objective of this work was to study the effect of supplementing melatonin alone during IVM in a defined culture medium on meiosis resumption and progression as well as its cytoprotective effects on bovine cumulus-oocyte complexes (COCs). Effect on subsequent embryo development was also addressed.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (Belo Horizonte, MG, Brazil) unless otherwise indicated.

2.2. Cumulus-oocyte complexes collection and IVM

Bovine ovaries were transported from the abattoir to the laboratory in sterile saline (0.9% NaCl) at approximately 30 °C within 5 hours after slaughter. The cumulus-oocyte complexes were manually aspirated from follicles 2 to 8 mm in diameter with an 18-gauge needle attached to a disposable 10-mL syringe. Cumulus-oocyte complexes with at least three compact layers of cumulus cells and uniform cytoplasm (Grades 1and 2) were selected [25]. To avoid confounding effects of serum and hormones, COCs were cultured in defined maturation medium, constituted of tissue culture medium 199 (TCM-199) supplemented with 0.25-mM sodium pyruvate, 0.1% polyvinyl alcohol (PVA), and 25-μg/mL gentamicin. Groups of 20 to 30 COCs were placed in 100-μL droplets under mineral oil and incubated for up to 24 hours at 38.5 °C in an atmosphere of 5% CO_2 in air and high humidity. For the experiments, the maturation medium was supplemented with 0.5-μg/mL FSH (Sigma) as a positive control or melatonin (10^{-9} and 10^{-6} M) as the experimental groups. A fourth group was matured without the addition of FSH or melatonin (negative control).

2.3. Assessment of oocyte nuclear maturation

To assess nuclear maturation resumption and progression, oocytes were evaluated for nuclear maturation stage after 6, 12, 18, and 24 hours IVM. Cumulus-oocyte complexes matured for 6 and 12 hours were stained with a primary antibody against nuclear envelope proteins lamin A and C conjugated with 4′,6-diamidino-2-phenylindole (anti-lamin A/C-DAPI) to detect the presence of the nuclear envelope and chromatin to follow meiosis resumption, whereas those matured for 18 and 24 hours were stained with Hoechst 33342 to determine maturation progression to metaphase II (MII). Before staining, oocytes were denuded, first by pipetting followed by vortexing for 3 minutes in 1% PVA solution (wt/vol) in Ca^{+} and Mg^{+}-free Dulbecco's phosphate-buffered saline (DPBS, Invitrogen). For anti-lamin A/C-DAPI staining, the denuded oocytes were fixed in 4% paraformaldehyde (wt/vol) in DPBS for 30 minutes. After fixation, oocytes were permeabilized with 0.5% Triton X-100 (v:v) in DPBS for 30 minutes, followed by 0.05% Tween 20 (v:v) in DPBS for another 30 minutes. Next, oocytes were incubated in blocking buffer (2% BSA (wt/vol) in DPBS) for 1 hour and then incubated in mouse anti-lamin A/C (1:300 in blocking buffer, Santa Cruz Biotechnology) for 1 hour, washed three times in DPBS, 5 minutes each and incubated in secondary antibody Alexa Fluor 488 labeled anti-mouse IgG (1:200 in blocking buffer, Santa Cruz Biotechnology) for 1 hour in the dark. Oocytes were then transferred through three 5-μL droplets Vectashield Mounting Medium containing DAPI (H-1200; Vector Laboratories), mounted on a glass slide under a coverslip using paraffin-vaseline support at each corner of the coverslip, and examined under an epifluorescence microscope (Nikon, Eclipse NI-U). Alexa Fluor 488 and DAPI stains were excited at 495 nm and 350 nm, respectively, to detect the presence of the nuclear envelope and chromatin. Nuclear status was classified into germinal vesicle (GV; nuclear envelope present) and metaphase I (MI; after nuclear envelope breakdown) stages [26]. For Hoechst staining, the denuded oocytes were stained with 10 μg/mL Hoechst 33342 in 1 mL PBS for 15 minutes in the dark and then

mounted onto a glass slide under a coverslip as described. Nuclear status was classified into MI and MII, and MII oocytes were considered as matured.

2.4. RNA isolation and real-time quantitative PCR

After 24 hours IVM, cumulus cells and oocytes were mechanically separated from pools of 20 COCs cultured per group by repeated pipetting in PBS/PVP, which were then collected separately. The denuded oocytes and corresponding cumulus cells were washed twice in DPBS with 1U/μL RNAse OUT (Recombinant Ribonuclease Inhibitor, Invitrogen) and stored at −80 °C until RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen. Inc) and quantified by spectrophotometry using a NanoDrop 2000 (Thermo Scientific). Total RNA (1 μg/reaction of cumulus cells or 20 oocytes) was incubated with DNase I according to the manufacturer's protocol. Complementary DNA was synthesized using a High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). Quantitative PCR reactions consisted of 20 μL containing 200 nM of each primer and 1x Power SYBR Green Master Mix (Life Technologies) and were performed in a thermocycler for real-time PCR (Applied Biosystems 7500 Real Time PCR System). The levels of accumulated fluorescence were analyzed using the second derivative method after the melting-curve analysis was complete, and then the expression levels of the target genes were normalized to the expression level of endogenous controls β-Actin and glyceraldehydes-3-phosphate dehydrogenase in each sample [27]. The conditions for real-time PCR were as follows: initial step at 50 °C for 2 minutes, followed by denaturation at 95 °C for 10 minutes and 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The dissociation curve was initiated at 60 °C with +0.1 °C increments up to 95 °C. The primer pairs for the analyzed messenger RNAs (mRNAs) are listed in Table 1.

2.5. Assessment of nuclear fragmentation in cumulus cells

Nuclear fragmentation was assessed in cumulus cells after IVM by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling reaction medium) assay followed by flow cytometry for quantification of cells presenting indications of cell death. TUNEL assay was performed with an *In Situ* Cell Death Detection Fluorescein Kit (Roche Diagnostics, Tokyo), according to the manufacturer's protocol. In brief, COCs were washed three times in 1% PVA solution and denuded by pipetting for the isolation of cumulus cells. The cells were transferred to a microtube and centrifuged at 1200 × *g* for 10 minutes at 21 °C. The pellet was then resuspended in 300 μL fixative solution (4% paraformaldehyde) and stored at 4 °C for 24 hours. Cells were permeabilized with 0.5% Triton X-100 and 0.1% sodium citrate in DPBS for 1 hour at 4 °C. Cells were separated in three groups: positive control (Cont+), negative control (Cont−) and experimental samples. The positive control was treated with DNase solution followed by TUNEL solution, the negative control was resuspended with enzyme-free marker solution and the other groups (experimental treatments) with the TUNEL solution. All groups were incubated for 1 hour in the dark in a wet chamber at 37 °C. The samples were then washed and stained with Hoechst 33342 (1 mg/mL) for 10 minutes, followed by washing and elution in 100-μL DPBS for analysis by flow cytometry (FACSAria, Beckton Dickinson Ltda) using the software FACSDiva v.6.2.1 (excitation 488 nm for annexin and 375 nm for Hoechst). At least 10,000 cells per group were analyzed.

2.6. Detection of reactive oxygen species (ROS) in oocytes

The determination of ROS levels in oocytes was performed using the methodology described by Frey et al. [28]. Cumulus-oocyte complexes were denuded after IVM and incubated with 10^{-5}-M carboxy-H_2 difluorofluorescein diacetate for 1 hour. After incubation, oocytes were fixed in 4% paraformaldehyde for 30 minutes and mounted on a glass slide under a coverslip with ProLong. The samples were examined using a microscope equipped with epi-fluorescence (Nikon, Eclipse NI-U model), using the filter with emission and/or excitation of 492 to 495 and/or 517 to 527 nm. Fluorescence intensity (f.i.) was measured using the Image J software (NIH; http://rsb.info.nih.gov/ij/) with intensity values set between 0 and 255 for each pixel.

2.7. In vitro fertilization and embryo culture

To evaluate the effects of treatments on embryo development, oocytes were *in vitro* matured in the same treatments as the other experiments (FSH and two melatonin concentrations), except for the negative control group, which was substituted for a laboratory *in vitro* production control group. This control group was matured in complete

Table 1
Primer sequences for analysis of transcript abundance.

Genes	Primer sequence (5′-3′)	Fragment size (bp)	Genbank accession no.
GAPDH	F: GGCCTCCAAGGAGTAAGGTC R: AGGAGATTCTCAGTGTGGCG	112	NM_001034034.2
β-Actin	F: GCAGGAGTACGATGAGTCCG R: TAACGCAGCTAACAGTCCGC	72	NM_173979.3
Cu,ZnSOD	F: ACACAAGGCTGTACCAGTGC R: TGTCACATTGCCCAGGTCTC	105	NM_174615.2
MnSOD	F: TCCTGTTCAATCGCAGTTACAGA R: ACGGGGTGGTGACTATCAGA	162	NM_201527.2
GPX4	F: TGTGGTGAAGCGGTATGGTC R: CACGCCAGGTTCTCAGGTCT	192	NM_174770.3

Abbreviations: F, forward; R, reverse; SOD, superoxide enzyme.

IVM medium (TCM199 supplemented with 10% FCS, 5-μg/mL FSH, 50-μg/mL LH, and 0.1-μg/mL estradiol). All other culture conditions for maturation were the same as previously described. After IVM, COCs were washed in IVF medium and placed in 70 μL droplets of the same medium. The frozen semen samples of the same bull were prepared by Percoll gradient technique (90% and 45%) to obtain motile sperm after removing the diluent and seminal plasma. Sperm cells in 30 μL IVF medium were added to the COCs droplet to a final concentration of 2×10^6 spermatozoa/mL. The IVF medium was modified Tyrode's medium plus penicillamine, hipotaurine, and epinephrine solutions and 10-μg/mL heparin. The gametes were coincubated in droplets covered with mineral oil for 18 to 20 hours at 38.5 °C in 5% CO_2 in air. After incubation, the oocytes were washed and transferred to droplets of culture medium (modified CR2 medium supplemented with 6 mg/mL BSA and 2.5% FCS) where they remained for up to 10 days. On the third day of IVC (Day 3), half of the medium was replaced with fresh IVC medium. At this moment, the proportion of cleaved oocytes (the number of embryos with two or more cells divided by total number of oocytes placed in culture) was also recorded. The blastocyst rate (the total number of blastocysts divided by number of oocytes placed in culture) was recorded on the eighth day (Day 8) and hatching rates (total number of hatched blastocysts divided by number of blastocysts on Day 8) on the 10th day (Day 10).

2.8. Experiments

2.8.1. Effect of melatonin during IVM on meiosis resumption and progression to metaphase II

In this experiment, COCs were matured *in vitro* in the different groups (control, FSH, 10^{-9} and 10^{-6} M melatonin) for 6, 12, 18, or 24 hours. At the end of culture, oocytes were denuded from cumulus cells and assessed for nuclear maturation status. Cumulus-oocyte complexes (n = 1081) were cultured and analyzed in four replicates (15–20 oocytes/treatment/replicate).

2.8.2. Effect of melatonin during IVM on the relative abundance of transcripts for antioxidant enzymes in oocytes and cumulus cells

Pools of 20 COCs/treatment/replicate were matured for 24 hours in the same groups as in the previous experiment. At the end of culture, COCs were separated in oocytes and cumulus cells, which were then prepared for PCR analyses. This experiment was repeated three times.

2.8.3. Effect of melatonin during IVM on nuclear fragmentation in cumulus cells

In this experiment, COCs (30–40 COCs/treatment/per replicate) were *in vitro* matured in the previously mentioned treaments for 24 hours. At the end of culture, oocytes were denuded to obtain cumulus cells. The cells were then prepared for TUNEL and flow cytometry to count apoptotic cells. This experiment was performed four times.

2.8.4. Effect of melatonin during IVM on levels of ROS in oocytes

For this experiment, COCs were matured for 24 hours in the same groups as previously described. At the end of culture, oocytes were denuded and analyzed for ROS levels. Oocytes (n = 286) were evaluated in four replicates (16–21 oocytes/treatment/replicate).

2.8.5. Effect of melatonin during IVM on subsequent embryo development in vitro

To evaluate the effects of melatonin during IVM on embryo development, COCs (n = 590) were matured *in vitro* in the same groups as before, except for the control which consisted of complete IVM medium (including hormones and serum), for 24 hours. At the end of culture, COCs were fertilized *in vitro*, and then embryos from the different IVM groups were cultured *in vitro* and assessed for cleavage, blastocyst development, and hatching rates. This experiment was performed four times (30–40 COCs/treatment/replicate).

2.9. Statistical analysis

To evaluate oocyte maturation and embryo development rates according to treatments, as well as ROS levels in oocytes (fluorescence intensity in arbitrary units), a completely randomized design was adopted using a generalized linear model (GLIMMIX procedure) for binomial data with a logit link function. For transcripts relative abundance, a completely randomized design, in 4×2 factorial, according to treatments and cell types was performed using a general linear mixed model (MIXED procedure). For these analyses, because significant effects were observed for the double interaction, the comparisons were conducted using Tukey test within each cell type. All tests were performed with the aid of the Statistical Analysis System [29]. Data are presented as the mean ± SEM.

3. Results

3.1. Effect of melatonin during IVM on meiosis resumption and progression to MII

In the first experiment, the effect of melatonin during IVM was evaluated regarding meiosis resumption (6 and 12 hours) and progression (18 and 24 hours). At 6 hours IVM, 100% of oocytes in all groups were immature and found in GV stage. After 12 hours, melatonin treatments (10^{-9} and 10^{-6} M) and FSH showed similar proportions of oocytes resuming meiosis and reaching MI stage varying from 69.6% to 81.1% between groups ($P > 0.05$; Fig. 1A). Control oocytes had lower MI rates (52.3%, $P < 0.05$) and, therefore, higher proportions of oocytes still in GV (47.7%, $P < 0.05$).

After 18 hours IVM, most oocytes had reached MII stage (58.2%–74.7%, $P > 0.05$; Fig. 1B). At 24 hours IVM, oocytes were also mostly in MII stage, and the highest proportion of matured oocytes observed in the FSH group (90.0%, $P < 0.05$), whereas the other groups had similarly lower proportions of MII oocytes (51.6%–69.1%, $P > 0.05$; Fig. 1C).

3.2. Effect of melatonin during IVM on the relative abundance of transcripts for antioxidant enzymes in oocytes and cumulus cells

In the second experiment, transcripts relative abundance for antioxidant enzymes in oocytes and cumulus

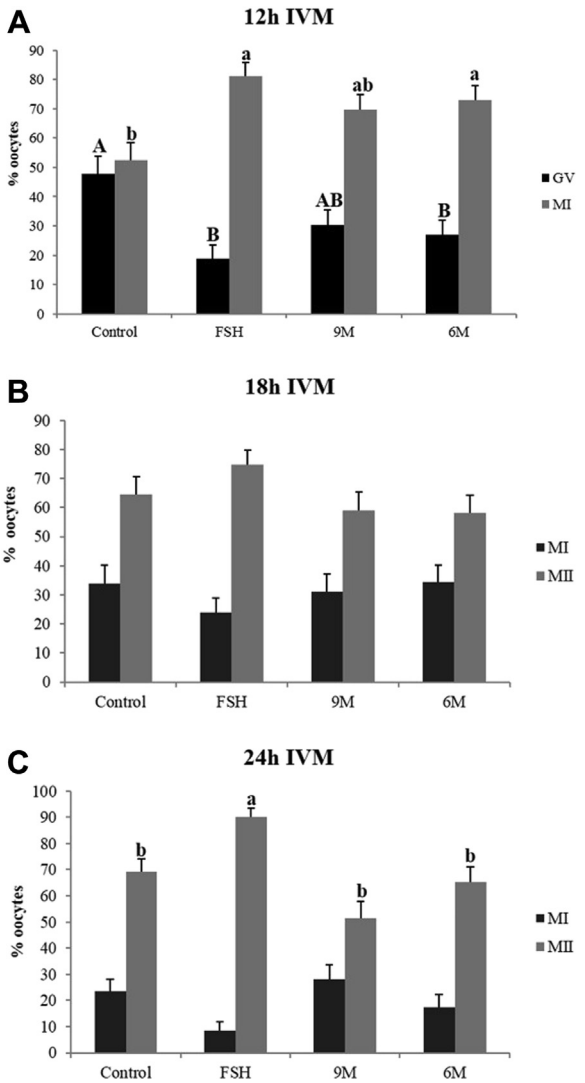

Fig. 1. Germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) rates in control, FSH, 10^{-9} M (9M), and 10^{-6} M (6M) melatonin groups at 12 hours (A), 18 hours (B), and 24 hours (C) IVM. ^{AB}Indicate differences between groups for GV stage. ^{ab}Indicate differences between groups for MI or MII stages ($P < 0.05$). Results are from four replicates.

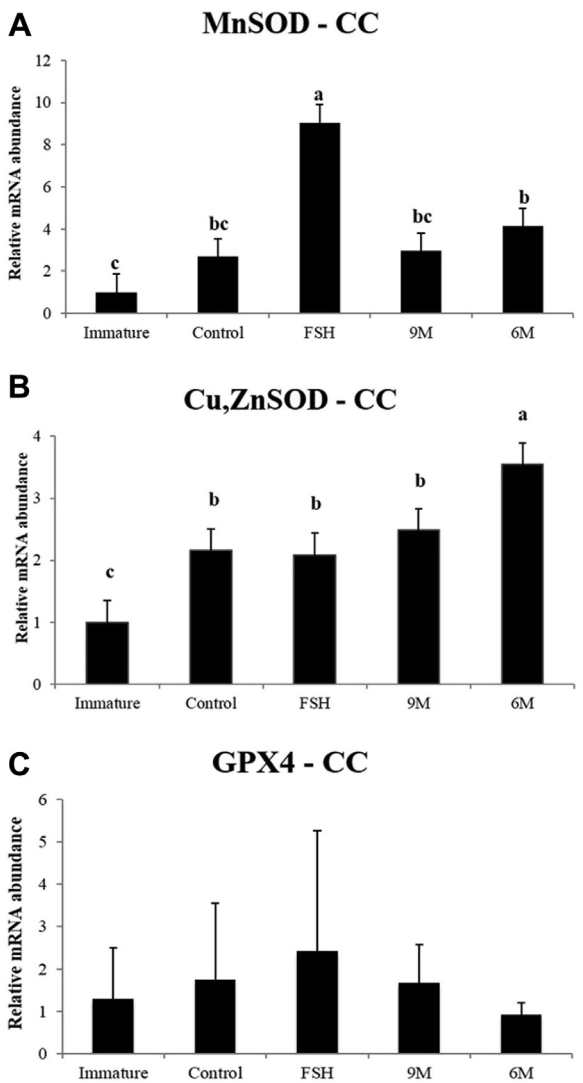

Fig. 2. Relative abundance of transcripts for *MnSOD* (A), *Cu,ZnSOD* (B), and *GPX4* (C) genes in cumulus cells at 0 hours (immature) and 24 hours IVM in control, FSH, 10^{-9} (9M), and 10^{-6} M (6M) melatonin groups. ^{abc}Different letters indicate statistical differences ($P < 0.05$). Results are from three replicates. CC, cumulus cell; mRNA, messenger RNA; SOD, superoxide enzyme.

cells immature or *in vitro* matured in the presence or absence of hormones were evaluated (Figs. 2 and 3). There was no effect of treatments on the expression of antioxidant enzymes in oocytes for any of the genes ($P > 0.05$). However, in cumulus cells, maturation with 10^{-6} M melatonin increased *MnSOD* transcripts relative to immature cells ($P < 0.05$), and the expression was highest in FSH compared with the groups matured with melatonin, without hormones, or immature cells ($P < 0.05$). Both concentrations of melatonin were similar to control group, and the lowest concentration and the control were also similar to the immature group ($P > 0.05$). Transcripts for *Cu,ZnSOD* were more abundant in cumulus from COCs matured with the highest melatonin concentration (10^{-6} M) in relation to immature cells and to the other maturation groups ($P < 0.05$). Control, FSH and 10^{-9}-M melatonin were similar among themselves ($P > 0.05$) but had increased transcripts when compared to immature cells ($P < 0.05$). *GPX4* was not affected by treatments or maturation ($P > 0.05$).

3.3. Effect of melatonin during IVM on nuclear fragmentation in cumulus cells

In the third experiment, the proportion of cumulus cells with nuclear fragmentation in the different treatments was examined. This parameter was affected by treatments, in which culture without addition of hormones had the highest proportion of nuclear fragmentation (37.4%/10,000

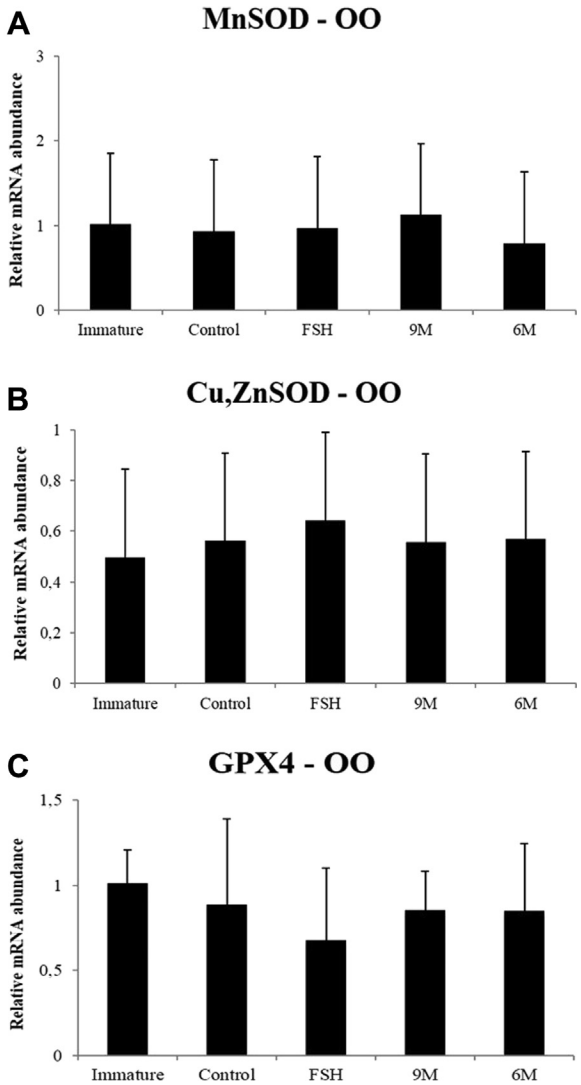

Fig. 3. Relative abundance of transcripts for *MnSOD* (A), *Cu,ZnSOD* (B), and *GPX4* (C) genes in oocytes at 0 hours (immature) and 24 hours IVM in control, FSH, 10^{-9} (9M), and 10^{-6} M (6M) melatonin groups. $P > 0.05$. Results are from three replicates. mRNA, messenger RNA; OO, oocytes.

cells, $P < 0.05$). Maturation in medium supplemented with 10^{-9}-M melatonin reduced fragmentation (30.8%/10,000 cells) but did not differ from control (no hormones) or the other maturation groups ($P > 0.05$). FSH and 10^{-6}-M melatonin similarly ($P > 0.05$) reduced fragmentation (29.4 and 25.6%/10,000 cells, respectively) relative to the control group ($P < 0.05$; Fig. 4).

3.4. Effect of melatonin during IVM on levels of ROS in oocytes

The effect of melatonin on ROS levels in oocytes was assessed on the fourth experiment. In this case, the fluorescence intensity as indicative of ROS levels in oocytes was affected by the treatments (Table 2). Control, FSH, and 10^{-9}-M melatonin were all similar among themselves (45.18–46.55 f.i., $P > 0.05$), but 10^{-6}-M melatonin

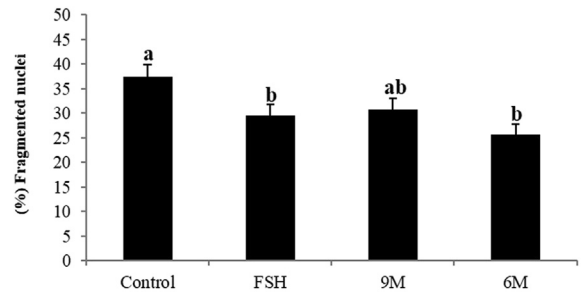

Fig. 4. Nuclear fragmentation in cumulus cells in control, FSH, 10^{-9} (9M), and 10^{-6} M (6M) melatonin groups from COCs matured *in vitro* for 24 hours. [ab]Different letters indicate statistical differences ($P < 0.05$). Results are from four replicates. COCs, cumulus-oocyte complexes.

(43.52 f.i.) was lower than control and FSH ($P < 0.05$) and similar to the lower concentration of melatonin (10^{-9} M, $P > 0.05$).

3.5. Effect of melatonin during IVM on subsequent embryo development in vitro

The effects of melatonin (10^{-9} and 10^{-6} M) and FSH during IVM were analyzed on subsequent embryonic development after *in vitro* fertilization and embryo culture in experiment 5 (Table 3). There were no differences between the groups matured either in complete medium (control) or in defined medium supplemented with any of the hormones tested (FSH and both melatonin concentrations). The developmental rates found were as follows: 82.0% to 87.3% cleaved embryos; 48.7% to 53.9% blastocysts; and 90.5% to 96.1% hatched ($P > 0.05$).

4. Discussion

Considering the cytoprotective role of melatonin including antioxidant and antiapoptotic functions and its beneficial effects during IVC, we have evaluated the effect of its supplementation in a defined culture medium for IVM, on oocyte nuclear and cytoplasmic maturation, and some cellular protection parameters in bovine oocytes and cumulus cells.

Regarding nuclear maturation, the proportions of oocytes undergoing meiosis resumption and progression to the metaphase II stage were determined at different time points along IVM culture. Cumulus-oocyte complexes matured with melatonin showed MI rates at 12 hours IVM,

Table 2
Fluorescence intensity of reactive oxygen species (ROS) levels detected by carboxy-H_2 difluorofluorescein diacetate staining in oocytes at 24 hours IVM in control, FSH, 10^{-9} (9M), and 10^{-6} M (6M) melatonin groups.

Maturation treatment	Oocytes (n)	Fluorescence intensity (arbitrary units); mean ± SEM
Control	64	45.78 ± 0.7^{a}
FSH	85	46.55 ± 0.6^{a}
10^{-9}M Melatonin	65	45.18 ± 0.6^{ab}
10^{-6}M Melatonin	72	43.52 ± 0.6^{b}

[ab]Different letters within the same column indicate significant difference between treatments ($P < 0.05$). Results are from four replicates.

Table 3
Embryo development of oocytes matured *in vitro* in the absence of hormones (control group) and presence of FSH or different melatonin concentrations.

Maturation treatment	Oocytes (n)	Embryo development		
		Cleavage, n (% ± SEM)	Blastocyst, n (% ± SEM)	Hatched blastocysts, n (% ± SEM)
Control	165	144 (87.3 ± 2.6)	89 (53.9 ± 3.9)	85 (95.5 ± 2.2)
FSH	121	101 (82.0 ± 3.1)	63 (48.7 ± 4.1)	68 (93.2 ± 3.0)
10^{-9}M Melatonin	152	127 (83.6 ± 3.0)	74 (48.7 ± 4.1)	67 (90.5 ± 3.4)
10^{-6}M Melatonin	152	132 (86.8 ± 2.7)	76 (50.6 ± 4.1)	73 (96.1 ± 2.2)

Cleavage (Day 3) and blastocyst (Day 8) rates were calculated relative to total oocytes placed in IVM and hatching rates (Day 10) relative do blastocysts in Day 8. There were no differences between treatments ($P > 0.05$). Results are from four replicates.

indicative of meiosis resumption, similar to oocytes treated with FSH at the same time point. Follicle-stimulating hormone is a known stimulator of oocyte maturation and frequently used in IVM procedures [30]. Therefore, the results indicate that melatonin is able to induce meiosis resumption at levels similar to the gonadotropin. Besides, as the medium was defined, that is, without addition of serum, which contains several variable and undefined components [31], the effects observed appear to be given by the indoleamine. Also, the observation that control oocytes cultured without any hormones as an indicator of solely spontaneous maturation had the lowest meiosis resumption rates compared to FSH and 10^{-6} M, further supports the idea that the effect was given by the hormones (melatonin or FSH, which were similar) and not merely to spontaneous meiosis.

To our knowledge, there are no reports on the ability of melatonin alone to stimulate meiosis resumption in mammalian oocytes. Studies in carps, however, have shown that melatonin treatment alone [32] or before hormonal stimulation can accelerate meiosis resumption *in vitro* [32,33]. The mechanism by which melatonin could stimulate meiosis resumption is unknown but could be related with the activation of maturation promoting factor (MPF). In carps, acceleration in meiosis resumption was associated to an earlier increase in cyclin B and $p34^{cdc2}$ proteins (MPF components) and MPF activity when the combined treatment (melatonin prior to hormone stimulation) was used. Maturation promoting factor is a key controller of meiosis resumption and progression, so a similar mechanism could be occurring in bovine oocytes matured with melatonin. Besides this, cAMP levels in oocytes must decrease to activate MPF and induce maturation [34], and melatonin inhibits adenylate cyclase, therefore, reducing cAMP levels [7,35], so this mechanism could also be involved. Melatonin receptors are present in bovine oocytes and cumulus cells [20,36], and they are known to decrease cAMP levels [35]. This possibility has been suggested as a melatonin agonist increased maturation, whereas an antagonist decreased [36], indicating that melatonin receptors may be implicated in maturation control. Whether melatonin decreases cAMP and/or leads to MPF activation in bovine oocytes, however, remains to be determined.

Regarding meiosis progression and completion of nuclear maturation to MII, melatonin treatments resulted in lower proportion of oocytes in MII. This observation suggests that although meiosis resumption was similar to FSH, its completion was slower, and that gonadotropin support may be necessary. Reports on the ability of melatonin to promote maturation are conflicting. In the few studies in cattle, melatonin added during IVM showed no effect [21] or decreased nuclear maturation [22], as observed in the present study. In contrast, El-Raey et al. [20] observed that melatonin stimulated nuclear maturation. Differences in culture conditions may explain the different results between studies, as the presence of serum [21] or serum and hormones [22]. The study showing positive effect in bovine oocytes [20] used BSA and FSH together with melatonin, whereas in this study only PVA was supplemented to evidence the exclusive effect of melatonin. Melatonin concentrations were also different (about 10^{-7} M in [20] and 10^{-6} and 10^{-9} M in present study). Besides, maturation rates in the control group (BSA + FSH, 49%) in [20] were lower than in the similar group in this study (PVA + FSH, 90%), so the difference between FSH and melatonin in our study was evident. The melatonin groups in both studies, however, were not so different (FSH + melatonin 67–73% in [20] and 67% for 10^{-6}M in this study). One other study also reported increased maturation [36], but regarded as embryo development (cleavage and blastocyst rates) as MII rates were not analyzed. Besides, serum and hormones together with melatonin during IVM culture were also used [36].

In other species, the effect of melatonin on maturation is also variable with either no effect (humans [37], pigs [38]) or negative effect (pigs [38], humans [39]) but mostly positive effects (sheep [40]; buffaloes [41]; pigs [42]; and humans [39]). Studies in humans [39] and pigs [38] suggest that the effect of melatonin is concentration dependent. Variations in the composition of culture medium, supplementation with other additives such as serum, follicular fluid, hormones, among other factors, contribute to the varied results. Most studies included hormones, as well as serum or follicular fluid, known to contain melatonin [19].

Among the reported functions of melatonin, one of the most important is protecting cells against oxidative stress, directly as a free radical scavenger or indirectly by acting on the expression and activity of antioxidant enzymes [43]. Regarding transcript abundance for antioxidant enzymes, there were only variations in cumulus cells. Although there are no reports on the effect of melatonin on the expression of these antioxidant enzymes in oocytes and cumulus cells, this function of melatonin has already been reported in other cell types [43] and embryos [44–46]. In cumulus cells, maturation only affected the superoxide enzymes (SODs),

but different isoforms increased depending on the hormone present during culture. *Cu,ZnSOD* increased relative to immature cells when matured with the highest melatonin concentration (10^{-6}M), whereas *MnSOD* increased relative to immature cells with the same melatonin concentration, and further with FSH.

The proteins for the three SOD isoforms were detected in different compartments of the bovine ovarian follicle [47], in agreement with our results, except for MnSOD, which was not detected as protein in cumulus cells, but we detected the mRNA for this enzyme. Khalil et al. [48], however, also detected *MnSOD* as well as *GPX4* and *GPX1* transcripts in bovine COCs confirming our observations. Differences in detection method (Western blotting for proteins and real time PCR for mRNA) could explain the discrepancy between studies.

The effect of melatonin to increase *Cu,ZnSOD* transcripts, however, has not been shown before in cumulus cells. *In vitro* culture with melatonin also increased SOD in mice [44] and *Cu,ZnSOD* in bovine embryos [45,46], evidencing that melatonin has similar effects in cumulus cells. Kang et al. [49] have shown that *MnSOD* increases in pig cumulus cells after IVM in the presence of 20% O_2, which is similar to the conditions used in this study (5% CO_2 in air), so increased SODs expression is coherent with IVC conditions. Higher melatonin increased *MnSOD* relative to immature cells but was similar to the other matured groups; so, for this isoform, melatonin had limited effect. On the other hand, FSH further increased *MnSOD* relative abundance relative to all other groups. The relationship between FSH and antioxidant enzyme expression has not been reported before, except for a study showing an increase in *SOD3* when rat Sertoli cells were treated with FSH [50]. Although we did not study this isoform, Combelles et al. [47] have detected this protein in bovine cumulus cells, so this other isoform would be an interesting target for further studies. In contrast, Sutton-McDowall et al. [51] have shown that FSH reduces the expression of genes related to GSH recycling in bovine oocytes. So at this point, it is difficult to indicate the role of FSH on the expression of antioxidant enzymes in oocytes and cumulus cells warranting further examination.

Regarding *GPX4*, no effects were observed in cumulus cells or oocytes, but results confirm previous findings of Khalil et al. [48] and Sutton-McDowall et al. [51] of the presence of this enzyme, as well as the other isoform *GPX1* in bovine COCs. Melatonin effects have been reported in mice embryos, in which the *GPX1* isoform was not affected [44] and in bovine embryos in which *GPX4* was increased [45,46]. Culture conditions and cell types studied are probably responsible for variations in response reported in the different studies.

Increased oxidative stress caused by culture can lead to apoptosis, and melatonin is recognized as a potent antioxidant and is able to reduce apoptosis [7,43]. Nuclear fragmentation was lower in cumulus cells of COCs matured with melatonin (10^{-6}M), and this group had increased transcripts for *Cu,ZnSOD*, indicating that melatonin could protect cumulus cells against nuclear fragmentation by increasing SOD expression. Effects of melatonin on the expression of other proteins involved in apoptosis may also occur, as transcripts for antiapoptotic proteins were increased and those for proapoptotic decreased in embryos of several species (cattle [45]; mice [44,52]; and pigs [53]), although Rodríguez-Osorio et al. [54] did not observe such effects. Our results corroborate those of Takada et al 2012 [55], where DNA integrity measured by comet assay was preserved in cumulus cells from bovine COCs matured with melatonin. A study in mice [56] also showed similar results. Reduction in apoptosis by melatonin has also been reported for bovine denuded oocytes [57] and embryos [46], and embryos of other species (pigs [53,58]; mice [44,59]). Only one study in pig embryos reported no effect of melatonin [38]. Therefore, our results extend previous findings by showing that melatonin protects bovine cumulus cells from nuclear fragmentation during IVM and could be an important supplement not only to stimulate maturation but also to protect COCs from oxidative stress during IVC.

When analyzing ROS levels in oocytes, a similar trend was observed, and oocytes treated with the higher melatonin concentration had lower fluorescence intensity. There are few studies on the relationship between melatonin and ROS levels in bovine oocytes, but El-Raey et al. [20] also observed reduction in oocyte ROS levels after maturation with melatonin. Zhao et al. [57] reported partial reversion of increased ROS levels when denuded oocytes were matured with melatonin. Lord et al. [52] have observed lower ROS levels in aged-mice oocytes cultured with melatonin, and similar results were reported for embryos [38,42,46,58,59].

With respect to the effect of melatonin on embryo development, both melatonin concentrations showed similar results for cleavage, blastocyst, and hatching rates, when compared to groups matured in defined medium only with FSH and complete maturation medium (hormones and serum). Some studies have shown that melatonin during IVM culture can improve subsequent bovine embryo development [24,36] or has no effect [21,23,55], as observed in the present study. In other species, similar variation in outcomes has also been reported, with either no effects [40,58] or improvement in embryo development [18,42,52] and even decrease depending on concentration used [38]. Different culture media and concentrations used are probably related with such variations on results as mentioned previously. Most of these studies have used either serum or follicular fluid and hormones in different combinations, and in our study, IVM was carried out in defined medium containing only melatonin, and still, embryo development was similar to that of oocytes matured in standard IVM medium containing hormones and serum. Therefore, the results suggest that melatonin alone was able to support oocyte nuclear and cytoplasmic maturation to undergo fertilization and embryo development up to the hatched blastocyst stage at proportions similar to those observed for oocytes matured under standard culture conditions used in IVP systems. As shown in the previous experiments, melatonin alone was able to stimulate meiosis resumption and to protect cumulus cells from apoptosis, possibly due to the increased expression of antioxidant enzymes and reduced ROS levels in oocytes. Besides, although completion of maturation was lower at 24 hours, embryo cleavage and development was as efficient as standard maturation; so, maturation

progression was probably only delayed and not inhibited by melatonin. Cleavage rate can also indicate that maturation has occurred [36], and all groups had similar and high cleavage rates (about 80%). Taken together, our results demonstrate the potential for the use of melatonin on different aspects of the processes of *in vitro* embryo production and the need for further studies to clarify the role of melatonin on the function of COC to take advantage of its varied roles in cells to improve outcomes of assisted reproductive technologies.

In conclusion, although melatonin supplementation in defined maturation medium does not stimulate meiosis progression, it stimulates meiosis resumption in bovine COCs and such treated oocytes are capable of supporting subsequent embryo development *in vitro*. Melatonin also protects cumulus cells from nuclear fragmentation and increases the expression of antioxidant enzymes as well as decreases ROS levels in oocytes. The indolamine may affect different aspects of bovine COC functions during IVM, and its role must be explored further to improve the results of *in vitro* embryo production systems.

Acknowledgments

The authors are grateful for the financial support from the Brazilian research agencies FAPESP (grant # 201/18023-9) and CNPq (Maria Carolina Rodrigues-Cunha Master's scholarship).

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