Use of Melatonin in the *In Vitro* Production of Bovine Embryos

Uso da Melatonina na Produção In Vitro de Embriões Bovinos

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ABSTRACT

We aimed to assess the effects of melatonin in the *in vitro* production of bovine embryos. Our experiment was conducted at the Laboratório de Reprodução Animal of the Universidade Estadual do Maranhão. The cumulus-oocyte complexes (COCs) were distributed among treatments at concentrations of 0, 10^{-1}, 10^{-3} and 10^{-5} μMol/L melatonin. Our experiment was further divided into two: the first was to assess the effect of different concentrations of melatonin (treatments) on the maturation rate of COCs, and the second was to assess the effects of melatonin treatments on the *in vitro* production of bovine embryos. The results from the first experiment demonstrated no significant difference between the *in vitro* maturation rate of the cultivated COCs in treatments with melatonin. In the second experiment, however, melatonin treatments yielded statistically higher cleavage, morula and blastocyst rates in the 10^{-5} μM group (52.9%, 52.9%, and 35.3%, respectively), and lower rates in the 10^{-1} μM group (19.5%, 19.5% and 7.8%, respectively), compared to the others. The control group (no melatonin) and the 10^{-3} μM group showed similar results. We concluded that supplementation of melatonin in the *in vitro* maturation medium resulted in no improvement in the oocyte maturation rate, but in the *in vitro* production of embryos at different concentrations, the 10^{-5} μM group displayed better results, but with no improvement in the variables ($P < 0.05$).
Keywords: COC, Melatonin, IVP, Follicular Aspiration.

RESUMO

Objetivou-se avaliar os efeitos da melatonina na produção in vitro de embriões bovinos. O experimento foi conduzido no Laboratório de Reprodução Animal da Universidade Estadual do Maranhão. Os complexos cumulus-oócitos (CCOs) foram distribuídos entre os tratamentos 0, 10-1, 10-3 e 10-5 μmol/L de melatonina. A avaliação foi dividida em dois experimentos, onde o primeiro avaliou o efeito dessas diferentes concentrações de melatonina (tratamentos) sobre a taxa de maturação dos CCOs e o segundo, o efeito desses tratamentos com melatonina sobre a produção in vitro de embriões bovinos. Os resultados no primeiro experimento demonstraram não haver diferença significativa na taxa de maturação in vitro dos CCOs cultivados no tratamento com melatonina. No entanto, o tratamento com melatonina no segundo experimento, as taxas de clivagens, mórulas e blastocistos, o grupo 10-5 μM foi estatisticamente superior (52,9%, 52,9% e 35,3%, respectivamente) e o grupo 10-1 μM inferior (19,5%, 19,5% e 7,8%, respectivamente) aos outros grupos. O grupo controle (sem melatonina) e o grupo 10-3 μM obtiveram resultados semelhantes. Concluiu-se que a suplementação da melatonina no meio de maturação in vitro não evidenciou melhoras na taxa de maturação dos oócitos, porém na produção in vitro de embriões em diferentes concentrações, o grupo 10-5 μM apresentou melhores resultados mesmo não havendo melhorias nas variáveis (P<0,05).
Palavras-chave: CCOS, Melatonina, PIV, Aspiração folicular

INTRODUCTION

Biotechnologies are a set of techniques implemented to manipulate organisms and/or their components, associated with molecular biology, cloning and genetic engineering (Wetherington, 2010).

In vitro production (IVP) is a biotechnology that consists in recovering immature oocytes from ovarian follicles, thus allowing for the intensification of use of animals with high genetic value (Merton et al., 2003). The year 2017 is a historic landmark in the production of bovine embryos. For the first time in the records of the International Embryo Technology Society (IETS), the total number of IVP embryos surpassed those produced *in vivo* (992,289 versus 406,287, respectively) (Viana, 2018).

According to Thompson et al. (2007), several IVP methods have been employed to produce embryos from immature oocytes. Bovine IVP reaches average development rates of 20 to 40% until the blastocyte stage.

In the context of reproductive biotechnology, the quality and integrity of *cumulus*-oocyte complexes (COCs) influence the success of *in vitro* cultivation. Thus, oxidative stress is a relevant factor that impairs *in vitro* development of COCs and the fertilization and *in vitro* culture of the embryo. Melatonin is a product of the pineal gland, acting as a potent antioxidant and eliminating reactive oxygen species (ROS), altogether avoiding oxidative stress during fetal development. It also plays other roles, such as modulating the ovarian function in mammals (Tsantarliotou et al., 2007). In this way, it can effectively alleviate aging in *in vitro*-produced oocytes provoked by oxidative stress, thus delaying the beginning of apoptosis and avoiding cell fragmentation (Lord et al., 2013).

Some effects of melatonin are mediated through specific membrane receptors, but many seem to be based on its potential as a direct scavenger of free radicals (Tamura et al., 2012). The presence of heightened levels of melatonin in the pre-ovulatory follicular fluid points to its possible role in the acquisition of oocyte competence during the process of maturation, fertilization and fetal development (Tsantarliotou et al., 2007).

Our research aimed at assessing the effects of melatonin in the production of *in vitro* bovine embryos considering the morphological aspects of the maturation of COCs and fetal development.

MATERIALS AND METHODS

Our experiment was conducted at the Laboratório de Reprodução Animal (LABRA) of the Universidade Estadual do Maranhão (UEMA). Ovaries were collected at the Municipal DA Vital slaughterhouse, located at BR – 135, km 03, in São Luís, Maranhão, Brazil.

Viable COCs were distributed between treatments of 0, 10^{-1}, 10^{-3} and 10^{-5} µM melatonin during *in vitro* maturation in all experiments. Our experiment was then divided in two phases: the first assessed the effects of distinct melatonin concentrations (treatments) on the maturation rate of COCs and the second assessed the effects of melatonin treatments on the *in vitro* production of bovine embryos. We used a total of 100 COCs per treatment, during both phases.

The following methods and proceedings herein described were approved by the Ethics and Animal Experimenting Committee (CEEA) of the Veterinary Medicine major at UEMA, agreeing with protocol nº 14/2016, approved on August 5th 2016, to conduct the research by observing the standards of Animal Welfare

on CRMV resolution Nº 1000/2012 and Brazilian Law 11.794/2008.

Ovaries were transported to the laboratory in a thermal vessel with 0.9% physiological solution containing 10% gentamicin at a temperature of 37 ºC. At the laboratory, ovaries were washed with 0.9% physiological solution at 37 ºC, and COCs measuring between 2 and 6 mm were recovered by follicular aspiration, using 25 x 8 mm sterile needles (21 g) coupled to a 10 mL syringe. The follicular liquid obtained was then stored in 15 mL Falcon tubes in a water bath at 37 ºC for 20 min for sedimentation. The aspired follicular contents were deposited on 100 x 20 mm Petri dishes for further research under a stereomicroscope. The selected COCs were transferred to 30 x 10 mm Petri dishes containing maintenance medium (TQC Holding Plus) and classified according to their morphological quality as Grade I, II, III or IV (Leidfried & First 1979). Only COCs of Grades I and II were selected for maturation.

COCs were washed three times in drops containing *in vitro* maturation (IVM) medium in 100 x 20 mm Petri dishes, then transferred to micro drops containing 100 µL IVM medium in 60 x 15 mm Petri dishes, which were numbered and contained eight IVM drops covered in mineral oil (Sigma-Aldrich, USA) at a temperature of 38.8 ºC, in a gaseous atmosphere of 5% CO_2 for 24 h. This *in vitro* maturation protocol for COCs was used during the first and second phases. This treatment was applied to all samples.

Melatonin was diluted in DMSO (dimethyl sulfoxide) at a concentration of 1 mg/mL, then stored at –20 ºC in 1 mL cryogenic tubes. During the first phase, melatonin was added to a micro drop containing maturation medium, following the treatment concentrations. After 24 h, COCs were kept for 10 min in a NUNCLON® plate containing 400 µL denudation medium (1 mL TCQ Holding + 10 mg/mL hyaluronidase); then, COCs underwent mechanical agitation to remove cumulus cells, evaluated under a stereoscope, for extrusion of the first polar corpuscle.

In the second phase, COCs were matured and, after 24 h of maturation, were washed three times in 900 µL fertilization medium supplemented with 20 µL heparin and 40 µL PHE (penicillamine, hypotaurine and epinephrine) solution.

Semen was provided from a single Gir bull and batch. It was unfrozen in water at 37 ºC for 30 s, then deposited in a 1.5 mL Eppendorf over a 45% and 90% Percoll gradient submitted to a centrifuging force of 251g for 10 min. A 10 µL volume was removed to assess motility and initial vigor. After centrifuging, spermatozoids at the bottom of the Eppendorf were aspirated and put in 1 mL of *in vitro* fertilization medium, then resubmitted to a 251g centrifugation for 5 min. After the second centrifugation, the excess *in vitro* fertilization (IVF) medium was removed. A 10 µL sample was used for a final evaluation of sperm motility and vigor. Then, 5.0 µL of the suspension was removed and added to 95 µL water for determination of sperm concentration by sperm cell count in a Neubauer chamber. Final sperm concentration was adjusted to 25 million live spermatozoids/mL. Afterwards, COCs and spermatozoids were co-incubated at 38.8 ºC for 18 to 22 h, under 20% O_2/5% CO_2 saturated humidity air.

After *in vitro* fertilization, the presumed zygotes were washed three times in drops of synthetic oviduct fluid, deposited in 100 µL drops and covered in mineral oil. Cleavage rate was assessed three days after the beginning of fecundation (Day 0). Embryo development (morula rate, initial blastocyst, complete blastocyst and

expanded blastocyst) was assessed at the fifth and seventh days (Days 5 and 7) following the beginning of fecundation.

The entire experiment design was random with four treatments (0, 10^{-1}, 10^{-3} and 10^{-5} µM melatonin) with 100 repetitions (number of COCs). At the first phase, we assessed the effect of melatonin on oocyte maturation; at the second phase we assessed the effect of melatonin on the *in vitro* production. The variables studied during the first phase (maturation rate) and second phase (cleavage rate, total blastocyst rate and proportion of viable embryos,

proportion per development stage and embryo quality) were submitted to a normality test. Normal data and data normalized under mathematical transformations (logarithmic, arcsine) were then submitted to analysis of variance (ANOVA) or to the Student-Newman-Keuls (SNK) parametric test for comparison of means, at a probability of 5%. Qualitative data were compared using the $\chi 2$ test, considering $P < 0.05$. Our analyses were conducted using software Statistical Analysis System (SAS Institute Inc, 1997).

RESULTS AND DISCUSSION

Experiment 1

We used 453 viable COCs in our experiment, and then distributed 116, 101, 113 and 126 COCs to groups 0, 10^{-1}, 10^{-3} and 10^{-5} µM melatonin, respectively, as shown in Table 1.

Table 1. Oocyte maturation rate of oocytes cultivated *in vitro* in medium supplemented with different melatonin concentrations and total COCs used per treatment. Numbers followed by distinct uppercase letters differ by the $\chi 2$ test ($P < 0.05$).

Maturation Rate (µM)	0	10^{-5}	10^{-3}	10^{-1}
	49.14% $(57/116)^{A}$	29.27% $(36/126)^{B}$	45.13% $(51/113)^{A}$	17.82% $(18/101)^{B}$

Regarding the maturation rate of in vitro-cultivated COCs, we observed that there was a significant difference at 0 µM melatonin (49.14%) in comparison to those at concentrations of 10^{-1} µM (17.82%) and 10^{-5} µM (29.27%). However, there was no significant difference when compared to a concentration 10^{-3} µM (45.13%). These results corroborate those of Adona et al. (2008), where an addition of 100 µM melatonin in an *in vitro* maturation medium did not interfere with the maturation rate and on the fetal development stages. However, our results differ from studies in which 1 µM and 100 µM melatonin

concentrations increased fetal development in bovines (Tan et al., 1993).

Manjunatha et al. (2009), using 20 and 50 µM melatonin in maturation medium of buffalo oocytes, obtained higher oocyte maturation percentages (90.3% and 88.8%, respectively), reporting a stimulatory effect of melatonin on *in vitro* maturation, thus differing from our present study.

The ability to undergo germinal vesicle breakdown and progress to metaphase I is acquired at the beginning of the growth phase, but the ability to progress to metaphase II is acquired later (Motlik; Fulk, 1986; Thibault et al., 1987).

Studies have demonstrated that some factors can improve oocyte competence, thus significantly increasing the number of blastocysts produced *in vitro* (Sirard et al., 1998). Although we know the influence of culture media and conditions, the oocyte's own original constituents are responsible for controlling the ability to respond to the most appropriate culture conditions (Sirard, 2001).

Experiment 2

Rates of cleavage, morula and blastocysts from our study groups are represented in Table 2. We observed that, compared to the control group, the rate of cleavage, morulae and blastocyst of the 10^{-5} µM treatment group differed in a statistically positive way (P < 0.05) in comparison to other groups, while the 10^{-1} µM treatment group differed negatively (P < 0.05) in comparison to the other groups. However, no dose was effective in improving these variables.

Table 2. Rate of cleavage, morula and blastocysts produced *in vitro* from COCs originated from *post-mortem* ovaries collected in a slaughterhouse for embryo IVP, submitted or not to different melatonin treatments. Numbers followed by distinct lowercase letters differ by the χ2 test (P < 0.05).

	Number of COCs	Cleavage rate	Morula rate	Blastocyst rate
Control	83	32 (38.6^a%)	30 (36.1^{ab}%)	15 (18.1^b%)
10^{-5} µM	51	27 (52.9^a%)	27 (52.9^a%)	18 (35.3^a%)
10^{-3} µM	88	36 (40.9^a%)	31 (35.2^b%)	8 (9.1^b%)
10^{-1} µM	77	15 (19.5^b%)	15 (19.5^c%)	6 (7.8^b%)

Compared to melatonin treatments, groups with smaller doses, 10^{-5} µM and 10^{-3} µM, presented an increased cleavage rate compared to groups submitted to higher melatonin doses and to the control group. This reinforces the literature, in which it is observed that melatonin supplementation in an embryo culture medium at a concentration of 10^{-9} µM has a positive effect on the cleavage rate and on the number of swine blastocyst cells (Rodriguez-Osório et al., 2007).

Bovine IVP reaches average rates of 20 to 40% blastocyst rates (Thompson et al., 2007). This highlights the 10^{-5} µM treatment group as a good option, as the blastocyst rate in this group reached 35.3% and guaranteed better results than the control group, even if did not improve the variables.

Although the melatonin dose in this experiment was used before the *in vitro* maturation, according to Papis et al. (2007), the melatonin dose was used during *in vitro* culture of bovine embryos and was questionable regarding the doses of 10^{-4} to 10^{-7} µM melatonin in the medium.

These previous results agree with studies in which melatonin concentrations of 10^{-4} to 10^{-9} µM increased murine and bovine fetal development (Tan et al., 1993; Siu et al., 2006).

Fetal development rates based on the stop stage of the in vitro development are represented in Table 3. Considering the proportion of morulas, the 10^{-3} µM group was superior to the rate obtained by the 10^{-5} µM group and similar to the control and 10^{-1} µM groups (P < 0.05). Control, 10^{-1}

µM and 10^{-5} µM groups were similar (P < 0.05).

Table 3. Fetal development rate based on the stop stage of *in vitro* development from COCs originated from *post-mortem* ovaries collected from slaughterhouses for embryo IVP, submitted or not to different melatonin treatments, evaluated on Days 5 and 7 after Day 0. Numbers followed by distinct lowercase letters differ by the χ2 test (P < 0.05).

	Morula	Initial blastocyst	Blastocyst	Expanded blastocyst	TOTAL
Control	15 (50.0%)[ab]	7 (23.3%)[ab]	6 (20.0%)[a]	2 (6.7%)[a]	30
10^{-5} µM	9 (33.3%)[b]	12 (44.4%)[a]	4 (14.8%)[a]	2 (7.4%)[a]	27
10^{-3} µM	23 (74.2%)[a]	3 (9.7%)[b]	4 (12.9%)[a]	1 (3.2%)[a]	31
10^{-1} µM	9 (60.0%)[ab]	4 (26.7%)[ab]	1 (6.7%)[a]	1 (6.7%)[a]	15
TOTAL	56	26	15	6	

During the morula stage there is activation of the fetal genome, which may be considered one of the most crucial moments. This activation takes place when the mother transcripts are substituted, starting from the fetal genome, and then begin to direct the initial development. This complex requires nuclear and cytoplasmatic modifications (Lima & Sousa, 2009). According to Kanka (2003), changes in the structure of embryo chromatins at the initial stages exert a relevant role in genome activation but are not sufficient to activate transcription. In this context, other studies have observed that cytoplasmatic changes (mainly availability, content or activity of transcriptional factors) act together with chromatin changes for the success of transcription (Latham & Schultz, 2001). According to the stop stage of the fetal development, melatonin at a lower concentration (10^{-5} µM) has a smaller percentage (33.3%), thus presupposing that this quantity of melatonin aids in genome activation, decreasing the number of embryos that stagnate during the morula stage. This is unlike the 10^{-3} µM concentration, which obtained an inverse reaction and provoked an increased stagnation of embryos during this development stage.

These results disagree with those of Tan et al. (1993), where 1 pM and 100 µM melatonin concentration increased bovine fetal development.

In vitro fetal development rates (%) based on morphological quality are shown in Table 4. The proportion of Grade I was similar (P < 0.05) in the control and 10^{-3} µM groups; other groups did not present rates. For the proportion of Grade II, all treatments were similar (P < 0.05), but the 10^{-5} µM group did not present structures.

Table 4. *In vitro* fetal development rates (%) based on morphological quality (IETS, 2014) from COCs originated from *post-mortem* ovaries collected from slaughterhouses for embryo IVP, submitted or not to different melatonin treatments, evaluated on Days 5 and 7 after Day 0. Numbers followed by distinct lowercase letters differ by the $\chi 2$ test ($P < 0.05$).

	GRADE I	GRADE II	GRADE III	TOTAL
Control	1 (3.3%)[a]	5 (16.7%)[a]	24 (80.0%)[b]	30
10^{-1} μM	0	1 (6.7%)[a]	14 (93.3%)[ab]	15
10^{-3} μM	1 (3.2%)[a]	4 (12.9%)[a]	26 (83.9%)[b]	31
10^{-5} μM	0	0	27 (100%)[a]	27
TOTAL	2	10	91	

Regarding the Grade III quality, the 10^{-5} μM and 10^{-1} μM groups were similar, but the 10^{-5} μM group was superior ($P > 0.05$) to the control and 10^{-3} μM groups. Control, 10^{-1} μM and 10^{-3} μM groups were similar ($P < 0.05$).

According to Pocar et al. (2005), the quality of cumulus cells is considered to be a crucial factor that influences oocyte maturation and their subsequent development competence. However, even in the present study the lowest morphological quality (Grade III) showed a superior performance rate when submitted to 10^{-5} μM melatonin treatment.

On the results of the first phase, melatonin supplementation of the *in vitro* maturation medium did not show evidence of improvements in oocyte maturation rates. In the second phase, melatonin supplementation at different concentrations during the *in vitro* production of embryos was able to improve the post fecundation phase *in vitro*. The 10^{-5} μM group, in comparison to the other groups, presented higher cleavage, morula and blastocyst rates during the culture period. Thus, it is suggested that this treatment yields positive effects on the *in vitro* production of bovine embryos. However, higher melatonin concentrations, such as at 10^{-1} μM, resulted in negative effects on the presented rates when compared to the control group.

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