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Melatonin improves *in vitro* maturation and subsequent embryo development of caprine oocytes*

SURBHI AGARWAL[1], S D KHARCHE[2] and A K BHATIYA[3]

ICAR-Central Institute for Research on Goats, Mathura, Uttar Pradesh 281 122 India

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ABSTRACT

The aim of the present investigation was to study the effect of melatonin on *in vitro* maturation and subsequent embryo development of caprine oocytes. In experiment 1, 384 *in vitro* matured oocytes were selected and randomly divided in to two groups, viz. group 1 (control) wherein oocytes matured in maturation media without supplementation for 27 h in humidified atmosphere at 38.5°C with 5% CO_2 in CO_2 incubator while in group 2 oocytes matured in maturation media with 30 ng/ml melatonin supplementation. After 27 h of culture, nuclear maturation was observed in both groups using Hoechst dye. In experiment 2, 1,336 oocytes were randomly divided into two groups, viz. group 1 (641) wherein oocytes were matured in maturation media without melatonin while in group 2 (n=695) oocytes matured in maturation media with 30 ng/ml melatonin supplementation. After 27 h, oocytes of both groups were then subjected to *in vitro* fertilization.The rate of nuclear maturation in group 2 (30 ng/ml melatonin) was significantly higher than that of group 1 (control). Similarly, the cleavage rate and blastocyst formation from *in vitro* matured goat oocytes were significantly higher in group 2 than that of group 1. In conclusion, the result indicated that the supplementation of 30 ng/ml melatonin in maturation media improves the nuclear maturation and subsequent cleavage rates and blastocyst production from caprine oocytes.

Key words: Blastocyst, Caprine, Embryo development, *in vitro* maturation, Melatonin, Oocytes

The manipulation of embryos during *in vitro* culture at ambient oxygen concentrations carries the risk of exposure to high levels of reactive oxygen species and free radicals, which adversely affect early embryonic development. Melatonin (N-acetyl-5-methoxytryptamine) is the main product secreted by the pineal gland and it also acts as an antioxidant, free radical scavenger and anti-apoptotic producer of developmentally competent embryos.The oxidative stress could be decreased by the antioxidant or radical scavenger in *in vitro* culture medium resulting in stimulation of culture conditions. Melatonin has been successfully tested as an antioxidant for promoting *in vitro* embryo development in bovine (Manjunatha *et al.* 2009). Since melatonin can rapidly pass through cellular membranes, every cellular organelle is exposed to this chemical messenger. This hormone directly destroys free radicals and indirectly by stimulating the antioxidant enzymes and inhibition of per-oxidation enzymes such as nitric oxide synthetase, acts as antioxidants (Galano *et al.* 2011, Zeebaree *et al.* 2018).

In addition to its benefits to *in vitro* maturation and early embryo development, melatonin might have a beneficial effect on embryo pre-implantation development because of its capacity as a radical scavenger, to protect embryonic cells from oxidative stress.Therefore, the present experiment was undertaken to study the effect of melatonin on *in vitro* maturation and subsequent embryo development.

MATERIALS AND METHODS

All organic and inorganic chemicals were purchased from Sigma Chemicals Co. unless otherwise stated.

Experiment 1: Assessment of morphological and nuclear maturation rate of caprine oocytes at 0 ng/ml and 30 ng/ml melatonin.

Recovery of oocytes: Goat ovaries (149) were collected from the local abattoir at Agra and transported within 4 h to the laboratory in warm saline (35–37°C), containing 100 IU penicillin-G and 100 µg streptomycin sulfate per ml. Oocytes were retrieved by slicing of the goat ovaries. Recovered oocytes were graded as per Kharche *et al.* (2008).

In vitro *maturation:* Selected cumulus oocyte complexes (COCs) were washed 8 to 10 times in 50–100 µl drops of oocyte holding medium (OHM) and subsequently 2 to 3 times in 50 µl drops of oocyte maturation media.Oocytes were randomly divided into two treatment groups on the basis of concentrations of melatonin added i.e. group 1

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Present address: [1]Ph.D Scholar (subhi.agrawal89 @gmail.com),[3]Professor and Head (ak.bhatiya @gla.ac.in), Department of Biotechnology, GLA University, Mathura, Uttar Pradesh, India. [2]Principal Scientist (kharche62@gmail.com), Animal Physiology and Reproduction Division.

(control) and group 2 (30 ng/ml melatonin).

Group 1 (control) (227): Oocytes were matured in 50 µl drops of maturation media containing TCM-199 (Sigma), L-glutamine (100 µg/ml), sodium pyruvate (0.25 mmol), gentamycin (50 µg/ml), FSH (5 µg/ml), LH (10 µg/ml), oestradiol-17β (1µg/ml), EGF (10 ng/ml) supplemented with 10% FBS, 10% follicular fluid and 3 mg/ml BSA covered with sterile mineral oil for 27 h in humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator.

Group 2 (157): Oocytes were matured in 50 µl drops of maturation media supplemented with 30 ng/ml melatonin.

Evaluation of in vitro *matured oocytes:* In the present study, Hoechst dye was used to stain the oocytes for the observation of polar body and two chromatin spots that assured maturation.

Staining: After 27 h of maturation, oocytes were stripped off their cumulus cells by gentle pipetting for 1 min in 0.1% hyaluronidase enzyme. Denuded oocytes were then selected and washed in PBS (1×) followed by fixation with Paraformaldehyde for 10 min. Oocytes were then stained with Hoechst dye for 30 min in dark. Oocytes were then washed with 1× PBS and evaluated under an inverted phase-contrast microscope (Nikon, Tokyo). Nuclear stages were distinguished by the morphology of chromatin material as per Yadav *et al.* (2013). Oocytes with second metaphase plate and first polar body were classified as mature phase of second meiotic cell division (Figs 1 and 2).

Experiment 2: In vitro *embryo development of* in vitro *matured caprine oocytes in melatonin supplemented media*

Recovery of oocytes and *in vitro* maturation was done as described above. A total of 1,336 oocytes were isolated from 503 ovaries and were randomly divided into two groups viz. group 1 (control, 641) wherein oocytes were matured in maturation media without melatonin while in group 2 (695) oocytes were matured in maturation media supplemented with 30 ng/ml melatonin for 27 h in humidified atmosphere at 38.5°C with 5% CO_2 in CO_2 incubator. After 27 h, the oocytes of both the groups were then subjected to *in vitro* fertilization.

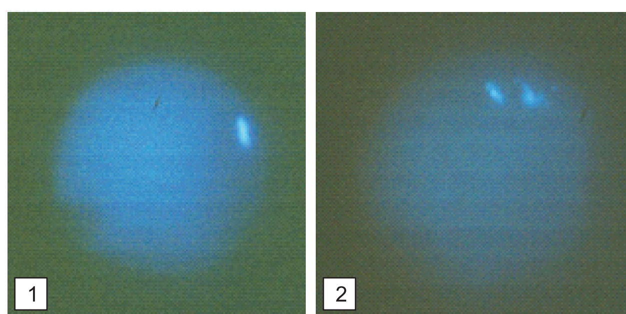
In vitro *fertilization:* Fresh semen was collected using the artificial vagina from a fertile pure breed adult Sirohi buck. *In vitro* fertilization was carried out as per the method described by Kharche *et al.* (2011) with slight modifications. Denuded oocytes from both groups were washed separately 10–20 times with Fert-TALP medium containing 10% FBS, 8 mg/ml fatty acid free BSA and 50 µg/ml heparin. Approximately 15–20 matured oocytes were transferred in each 50 µl drop of Fert-TALP medium and the drops were inseminated with 15–20 µl of the final diluted semen so as to obtain a sperm concentration of $1–2\times10^6$ sperm/ml. After *in vitro* insemination, the oocytes and sperm were co-incubated for 18 h at 38.5°C with 5% CO_2 in humidified atmosphere.

After 18 h of sperm oocytes co-incubation, sperms were washed in embryo development medium to remove extra sperm cells adhered to zona pellucida. Oocytes of both groups were finally transferred into 50 µl drops of embryo development medium, Research Vitro Cleave (RVCL) from Cook Medical, Australia (Readymade/complex culture medium) supplemented with 1% BSA for 48 h in humidified atmosphere of 5% CO_2 at 38.5ºC in CO_2 incubator. After 48 h of post insemination, fertilized oocytes were evaluated under phase contrast microscope for cleavage rate. Cleaved oocytes were cultured in embryo development media further for 8–10 days. The culture media was replaced after every 48 h and observations were taken for subsequent embryo development. The embryo development was observed under inverted phase contrast microscope up to 10 days.

Statistical analysis: The maturation stage of oocytes was calculated as a percentage. Cleavage rates and embryo development between the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

A total of 149 ovaries were collected for experiment 1 and an average recovery rate (grade A, B and C oocytes as per Kharche *et al.* (2008)) of 2.67±0.30 was observed. Furthermore, the rate of morphological maturation on the basis of cumulus expansion and nuclear maturation in group 1 (control) was 89.59±1.22% and 48.66±1.72%, whereas the rate of morphological maturation on the basis of cumulus expansion and nuclear maturation in group 2 (30 ng/ml melatonin) was 92.56±3.99% and 85.50±7.34%, respectively (Table 1).



Figs 1-2. 1. M II oocytes with polar body; **2**. M II oocytes with two chromatin spot

Table 1. Morphological and nuclear maturation rate of caprine oocytes at 0 ng/ml and 30 ng/ml melatonin.

Group no.	Concentration of melotonin (ng/ml)	Morphological maturation	Nuclear maturation
1.	0 (Control)	203/227[a] (89.59±1.22%)	111/227[a] (48.66±1.72%)
2.	30	146/157 [a] (92.56±3.99%)	133/157[b] (85.50±7.34%)

Values with different superscripts within columns differ significantly ($P<0.05$).

Table 2. *In vitro* embryo development of *in vitro* matured oocytes

Group	Total cleavage (%)	2 cell (%)	4 cell (%)	8–16 cell (%)	Morula (%)	Blastocyst (%)
Group 1	135/589[a]	25/135	38/135	41/135	22/135	9/31[a]
(IVF Control)	(27.94±7.27)	(16.34±5.68)	(22.18±4.92)	(39.49±5.63)	(16.23±5.56)	(18.87±8.53)
Group 2	203/657[b]	19/203	35/203	65/203	51/203	33/84[b]
(Melatonin + IVF)	(44.07±10.80)	(10.65±5.03)	(17.49±3.83)	(25.42±4.49)	(19.76±3.76)	(22.27±7.13)

Values with different superscripts within columns differ significantly (P<0.05)

Melatonin has the effect of inhibition of the activity of oxidative enzymes, stimulation of the activity of antioxidant enzymes, distribution in all tissue, cells and cellular compartments throughout the organism, and rapid diffusion through all biological membranes. During *in vitro* development of oocytes, cumulus cell provide nutrients and have an important role for growth and maturation of oocytes and also intervene the effects of hormones on oocytes. Additionally, expansion of cumulus cell is regarded as a chief indicator for maturation of oocytes and is important for further cleavage, and blastocyst development (Manjunatha *et al.* 2009, Galano *et al.* 2011, Zeebare *et al.* 2018). The results of this study showed that melatonin supplementation (30 ng/ml) to *in vitro* maturation medium had a potentially positive effect on the degree of cumulus cell expansion.When compared, our results of morphological maturation were superior to many workers (Kharche *et al.* 2007, 2008; Singh *et al.* 2009, Kharche *et al.* 2009, 2013; Kouamo and Kharche 2015) in goat. Our results were in agreement with Pathak *et al.* (2013) (95.03%) in caprine.

The present results of nuclear maturation with melatonin are superior to Kharche *et al.* (2005) (44.8%) in caprine and Magnus *et al*. (2010) (42.2%) in bovine, Rajabi-Toustani *et al.* (2013) (54.7%) in sheep.However, the small variation in maturation rate could be due to many reasons as maturation rate is influenced by many factors like presence of follicular cells, protein supplementation, hormones, antioxidants and growth factors in maturation media (Kharche *et al.* 2011).

Our result demonstrated that melatonin cannot improve cumulus expansion significantly. Melatonin improve oocyte meiotic maturation at metaphase-II stage. Therefore, melatonin supplementation during oocyte *in vitro* maturation may be used for combating the oxidative stress. Moreover, reduction of reactive oxygen species could be an aspect of the mechanism by which melatonin exerts its beneficial effects during caprine oocyte maturation.

The cleavage rate of *in vitro* matured goat oocytes and relevant data for embryo development are shown in Table 2. Our results of cleavage rate (*in vitro* fertilization) in control group were in agreement with Pathak *et al.* (2017) while cleavage rate following maturation with melatonin and subsequent embryo development without melatonin were higher than reported by Casao *et al.* (2010) (10^{-5} M and 10^{-6} M melatonin) in sheep oocytes (33.3% and 34.4%, respectively) and Niknafs *et al.* (2014) using 10 μM melatonin (30.6%) while our results were lower than Wang *et al.* (2014) (10^{-7} M melatonin) and Takada *et al.* (2010) (10^{-9} M melatonin) in bovine oocytes who stated better cleavage with melatonin when used both for oocyte maturation and embryo development media (87.78±1.02% and 85.7%, respectively).

Furthermore, our results of blastocyst production following maturation with melatonin and subsequent embryo development were higher than reported by Ishizuka *et al.* (2000) using 10^{-8} M and 10^{-4} M melatonin (17.5% and 12.5%, respectively) in mouse oocytes while our results were lower than Casao *et al.* (2010) (10^{-5} M and 10^{-6} M melatonin) in sheep oocytes (26.7% and 30.3%, respectively), Wang *et al.* (2014) (10^{-7} M melatonin) and Takada *et al.* (2010) (10^{-9} M melatonin) in bovine oocytes (38.33±2.21% and 42.8%, respectively). However, the results have not been decisive because of the use of different compounds and protocols and additional studies are needed to optimize the use of melatonin in IVM, IVF, and *in vitro* embryo culture procedures. Our results were in agreement with Shi *et al.* (2009) who reported that supplementing melatonin only in maturation medium promoted the cleavage rate and blastocyst production. 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 (Rodrigues-Cunha *et al.* 2016).

The result indicated that the supplementation of 30 ng/ml melatonin in maturation media improves the nuclear maturation and subsequent cleavage rates and blastocyst production from caprine oocytes.

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