B-4426
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RESEARCH ARTICLE Indian Journal of Animal Research

Role of Melatonin as a Survival Factor for *In vitro* Development of Sheep Preantral Follicles

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10.18805/IJAR.B-4426

ABSTRACT

Background: Melatonin, a powerful free radical scavenger and broad-spectrum antioxidant may directly affect ovarian function by regulating folliculogenesis, maintenance of follicular integrity, oocyte quality and maturation capacity. Therefore, we aimed to study effects of melatonin and its interaction with growth factors in sheep preantral follicles.

Methods: The influence of different concentrations of Melatonin (5-500 pM) on *in vitro* culture of preantral follicles (PFs') isolated from sheep ovaries was studied. Experiments I and II were conducted to standardize the optimum concentration of Melatonin that supports better development of preantral follicles. Experiment III was conducted with the optimum level of Melatonin derived in the Experiments I and II to evaluate the effect of melatonin at 100pM in combination with various growth factors.

Result: Overall follicular development was found to be the best in the PFs' cultured in medium supplemented with 100pM of Melatonin. Melatonin supplementation showed positive effects on the preantral follicular development in combination with different growth factors.

Key words: *In vitro* culture, Melatonin, Preantral follicles, Sheep.

INTRODUCTION

It was well established that mammalian preantral follicles do not grow *in vitro* as well as they do in *in vivo* (Arunakumari *et al.* 2010; Anil kumar *et al.* 2019). Two major reasons for such compromised development of preantral follicles are:1) Oxidative stress encountered during *in vitro* culture due to free radicals generated by endogenous process such as normal cellular metabolism and exogenous factors such as chemicals added to culture media, hyperoxia, exposure to light *etc.* (Natarajan *et al.* 2010) and 2) Frequent apoptosis of granulosa cells in culture. This indicates the need for presence of antioxidants in culture medium like Melatonin, a powerful free radical scavenger. Melatonin, a tryptophan containing hormone is synthesized predominantly in pineal gland, retina, gut and its production is also observed in the ovary in both cumulus cells and oocytes (Cassone *et al.* 1997; Soares *et al.* 2003). Earlier reports evidenced the presence of melatonin receptors in the ovarian follicular cells and follicular fluid (Soares *et al.* 2003) indicating the role of Melatonin in folliculogenesis (Taketani *et al.* 2011), oocyte quality and maturation capacity (Adriaens *et al.* 2006; Rodriguez *et al.* 2007) and maintenance of follicular integrity (Barros *et al.* 2013). Previous studies also indicated that melatonin was potentially useful in culture condition as it was a powerful free radical scavenger and a broad-spectrum antioxidant (Reiter *et al.* 2001; Nakamura *et al.* 2003; Tan *et al.* 2007) and it may directly affect ovarian function thereby serving as a local regulator in rats (Sakaguchi *et al.* 2013). Although existing information suggests that melatonin might control ovarian folliculogenesis, only meager reports are available indicating its direct role, especially on preantral follicles development to ovulatory stage in sheep. Accordingly the objective of the present study includes a) Standardization of optimum concentration of Melatonin that supports better development of sheep PFs' *in vitro* and b) Interaction of Melatonin with other hormones and growth factors and their influence on *in vitro* growth of sheep PFs'.

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How to cite this article: Kumar, C.C., Naik, B.R., Kumar, A.V.N.S., Ravi, A., Reddy, L.S.S.V. and Pathipati, D. (2021). Role of Melatonin as a Survival Factor for *In vitro* Development of Sheep Preantral Follicles. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4426.

Submitted: 15-02-2021 **Accepted:** 21-05-2021 **Online:** 16-09-2021

MATERIALS AND METHODS

Unless otherwise stated, culture media, hormones, growth factors, FCS and all the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All the methods described briefly hereunder are routinely employed in the culture of PFs' in the laboratory and described in detail in several earlier publication from the laboratory (Arunakumari *et al.* 2010; Kamalamma *et al.* 2016; Kona *et al.* 2016; Srividya *et al.* 2017; Anil kumar *et al.* 2019; Sravani pragna *et al.* 2020).

Isolation of preantral follicles (PFs')

A total of 175 ovaries and 790 preantral follicles collected on different days were used in this study. Ovaries recovered

after sheep slaughter were transported to the laboratory within 1h after slaughter in sterile, warm (37°C) phosphate buffered saline.

Intact preantral (PFs') in the size range of 250-400µm with intact basement membrane were mechanically isolated (Fig 1A) by micro dissection method from ovarian cortex under a stereo-zoom microscope (SMZ 2T, Nikon corporation, Japan) and cultured them for six days (Fig 1B, 1C, 1D) according to the methods developed in our laboratory.

Determination of optimum concentration of Melatonin for *in vitro* culture of Sheep PFs' (Experiments 1 and 2)

Preantral follicles were cultured in bicarbonate buffered tissue culture medium 199 supplemented with 50 mg/mL Gentamycin sulfate (TCM 199B) and different concentrations of Melatonin (M 5250) chosen on the basis of the results obtained in earlier studies on IVM of mouse oocytes (Nabiuni *et al.* 2013). Two separate experiments with different ranges of concentrations of Melatonin (Experiment: 1- 0, 5, 10, 25, 50, 100, 500 pM and Experiment: 2- 25, 50, 100, 250, 500 pM) were conducted.

Evaluating the effect of Melatonin in combination with other growth factors and hormones (Experiment 3)

To ascertain whether Melatonin supplementation to the culture medium encourage reasonable development and further improvement of cultured PFs' in sheep three differently supplemented media were used to culture the

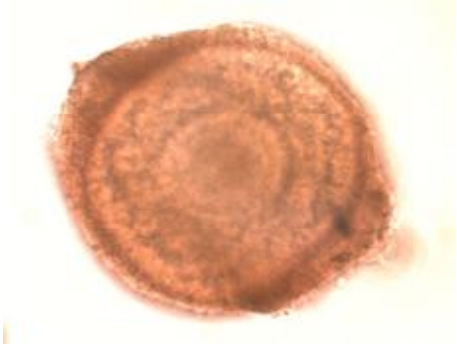

Fig 1A: Preantral follicle.

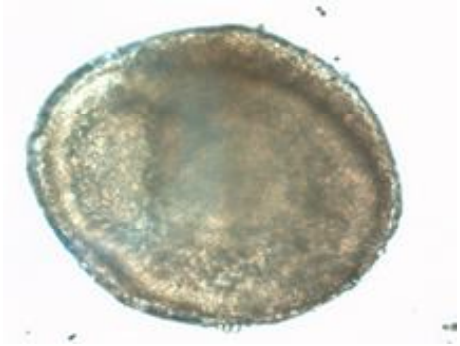

Fig 1B: Preantral follicle cultured for 2 days.

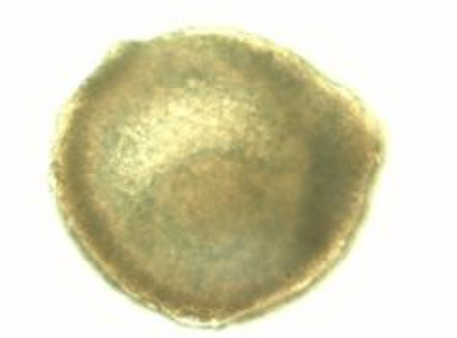

Fig 1C: Preantral follicle cultured for 4 days.

Fig 1D: Preantral follicle cultured for 6 days.

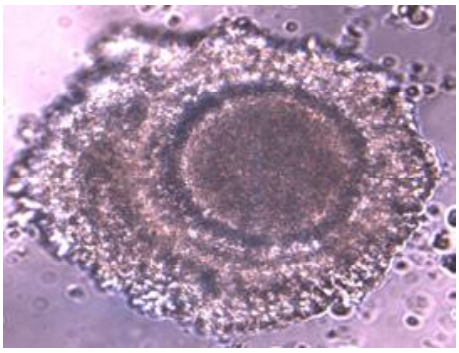

Fig 1E: Cumulus oocyte complex (COC) from 6 days cultured preantral follicle after subjecting to IVM for 24h.

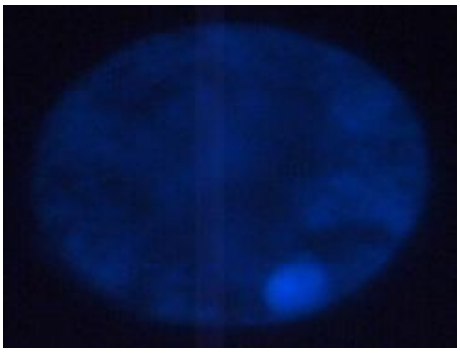

Fig 1F: M-II stage after IVM.

Fig 1A to 1F: Preantral follicle isolated from ovarian cortex (1A) and subsequently exposed to culture medium for two days (1B), four days (1C), six days (1D), Oocytes of COCs from six day cultured follicles further subjected to IVM for 24h (1E) and Metaphase –II stage of oocyte from cultured preantral follicles after IVM (1F).

PFs': (1) TCM 199 B medium containing 50 mg/mL gentamycin sulphate (control), (2) control medium supplemented with 1 mg/mL T4, 2.5 mg/mL FSH, 10 ng/mL IGF-1, 1 mIU/mL of GH (Arunakumari *et al.* 2010) (referred to as the standard medium hereafter) and (3) standard medium supplemented with 100 pM of Melatonin (M 5250).

Evaluation of growth and *In vitro* maturation of oocytes obtained from cultured PFs'

The cultured PFs' were morphologically evaluated every 24h for growth, increase in the diameter and antrum formation using an inverted microscope (Leica, DMIRB, Germany). The cumulus oocyte complexes from PFs' cultured for six days (Fig 1E) were further subjected to *In vitro* maturation for another 24h. Subsequently the oocytes were washed in Hoechst 33342 fluorescent stain solution (B 2261) and incubated in a 50µl droplet of the same solution for 15 minutes at 39°C (Rao *et al.*2002) which were examined under fluorescent light on an inverted microscope (Leica, Germany; excitation: 352-455nm and emission 460-490 nm) for M II stage (Fig 1F).

Statistical analysis

The dependent variables are the development parameters of the follicles, and independent variables are the different treatments in the experiments. Comparison of the proportion of PFs' exhibiting growth, average increase in diameter, antrum formation and maturation to MII stage of the oocytes from the *in vitro* cultured PFs' among different treatment groups were undertaken separately by one way ANOVA followed by Duncans multiple range test using SPSS 20 software.

RESULTS AND DISCUSSION

To our knowledge, this is the first study to address the influence of melatonin on *in vitro* development of preantral follicles. A total of seven hundred and ninety (790) preantral follicles were cultured *in vitro* in control, standard medium and melatonin treated groups. In experiment I, six different concentrations of melatonin (5-500pM) on *in vitro* development of PFs' were investigated individually and it appeared that melatonin at a concentration of 100pM favorably influenced the growth of PFs', average increase in diameter, exhibiting antrum formation and meiotic maturation percentage of oocytes to metaphase II from cultured PFs'. (Table 1). Previous studies by Shi *et al.* (2009) and El-Raey *et al.* (2011) support this observation wherein they indicated that higher levels of melatonin in the fluid of preovulatory follicles in porcine and bovine ovaries respectively might play an important role in process of ovulation. In contrast Ganji *et al.* (2015) reported that addition

Table 1: Effect of different concentrations of melatonin (0-500pM) on *in vitro* development of sheep preantral follicles.

Concentration of melatonin (Replicates/ No of follicles)	Proportion of PFs' exhibiting growth Mean±SE	Average increase in diameter (µm) Mean±SE	Proportion of PFs' exhibiting antrum formation Mean ±SE	Proportion of PFs' matured to MII stage Mean ± SE
Control (0pM)				
TCM 199B (10/50)	41.3±0.68^{b}	14.3±1.18^{a}	22.5±0.93^{b}	10.8+4.41^{a}
5pM (10/50)	13.8±0.94^{ab}	8.5±0.66^{ab}	8.6±0.92^{ab}	0.00
10pM (10/50)	32.8±1.09^{ab}	8.3±1.53^{b}	17.8±0.99^{b}	0.00
25pM (10/50)	42.3±1.12^{a}	12.3±0.77^{ab}	28.7±2.28^{a}	0.00
50pM (10/50)	60.1±0.88^{b}	20.1±0.65^{ab}	28.7±2.28^{b}	0.00
100pM (10/50)	69.6±5.17^{a}	21.6±0.92^{b}	51.6±1.78^{ab}	21.47±3.54^{c}
500pM (10/50)	29.2±1.49^{a}	14.3±0.89^{b}	40.5±1.81^{a}	0.00
Standard medium (10/50)	28.4±0.93^{a}	8.9±0.82^{a}	25.8±0.85^{ab}	14.6+8.43^{b}

Values (Mean±SE) with different superscripts within a column are significantly different. One way ANOVA followed by Duncan's Multiple Range Test (P≤0.05).

Table 2: Effect of different concentrations of melatonin (25-500pM) on *in vitro* development of sheep preantral follicles.

Concentration of melatonin (Replicates/ No of follicles)	Proportion of PFs' exhibiting growth Mean±SE	Average increase in diameter (µm) Mean±SE	Proportion of PFs' exhibiting antrum formation Mean ±SE	Proportion of PFs' matured to MII stage Mean ± S
TCM 199 B (Control; 10/50)	48.3±1.19^{ab}	6.4±0.718^{b}	34.5±5.24^{b}	10.1±3.5^{a}
25pM (10/50)	32.8±1.093^{a}	6.5±0.50^{a}	27.2±2.43^{ab}	0.00
50pM (10/50)	54.5±1.13^{a}	12.3±1.18^{ab}	43.8±3.51^{b}	0.00
100pM (10/50)	65.3±1.07^{ab}	14.5±1.03^{ab}	55.5±2.16^{ab}	20.8±4.1^{b}
250pM (10/50)	41.2±1.94^{b}	8.5±0.77^{a}	35.5±4.04^{a}	0.00
500pM (10/50)	41.2±1.94^{b}	10.2±0.89^{b}	34.9±5.31^{ab}	0.00

Values (Mean±SE) with different superscripts within a column are significantly different. One way ANOVA followed by Duncan's Multiple Range Test (P≤0.05).

Table 3: Effect of 100pM of melatonin in combination with standard medium on *in vitro* development of sheep preantral follicles

Concentration of melatonin (Replicates/ No of follicles)	Proportion of PFs' exhibiting growth Mean±SE	Average increase in diameter (µm) Mean±SE	Proportion of PFs' exhibiting antrum formation Mean ±SE	Proportion of PFs' matured to MII stage Mean ± S
TCM 199 B (Control 5/30)	45.3±1.19[b]	6.7±0.718[a]	34.2±5.24[b]	9.9±2.6[a]
Standard medium (5/30)	45.00±3.53[b]	10.8±0.77[b]	43.2±1.24[b]	14.7±4.1[b]
Standard medium+100 pM of melatonin (5/30)	79.6±6.44[a]	12.6±1.87[b]	58.8±2.81[a]	22.6±2.4[c]

Values (Mean±SE) with different superscripts within a column are significantly different. One way ANOVA followed by Duncan's Multiple Range Test (P≤0.05).

of 100pM melatonin in the culture medium had negative effect on their survival, growth and diameter in mouse which might be due to species variation. The proportion of PFs' exhibiting growth and average increase in diameter was similar in the PFs' cultured in TCM199B supplemented with 50pM and 100pM but the proportion of PFs' exhibiting antrum formation was significantly greater at 100 pM of Melatonin (Table 1). Although 25, 50 and 250 pM of melatonin supplementation appeared to induce greater increase in growth, diameter and higher proportion of the follicles exhibiting antrum formation, it was not statistically significant. Therefore another experiment involving the concentrations of 25, 50, 100, 250 and 500pM was undertaken. However, even in experiment II, 100pM of melatonin supported the best development thereby confirming that this might be the optimum concentration for *in vitro* development of preantral follicles (Table 2).

In our study, among the melatonin treatments only the oocytes collected from preantral follicles cultured in 100pM of melatonin reached to MII stage and interestingly 5, 10, 25, 50, 250 and 500pM concentrations of melatonin failed to support oocytes from cultured follicles to reach MII stage (Table 2). It may be speculated from previous studies that melatonin at 100pM could promote better expression of MT1 and MT2 receptors *via* c-AMP mediated signaling in the cumulus oocyte complexes or stimulation of maturation promoting factor of oocytes and in cumulus cells that positively affected the cytoplasmic and nuclear maturation (Soares *et al.* 2003; Chattoraj *et al.* 2005; El-Raey *et al.* 2011).

Further in Experiment III, 100pM concentration of melatonin was used in combination with growth factors and hormones to evaluate its action in inducing preantral follicular development with respect to parameters studied. While the average increase in diameter was similar in the PFs' cultured in standard medium and standard medium supplemented with 100pM of Melatonin, the proportion of PFs' exhibiting growth, antrum formation and meiotic maturation of oocytes to M-II were found to be significantly higher in standard medium supplemented with 100pM of Melatonin than in standard medium alone or in control medium (Table 3). It is remarkable to note that inclusion of melatonin (100pM) in the standard medium (*i.e.*, TCM 199B supplemented with FSH, T4, IGF-I and GH) supported better development and also resulted in highest proportion of oocytes reaching M-II from cultured follicles than in all other treatments. This could be due to the protective ability of melatonin on the cultured PFs' by modulating the growth factor expression (Schaeffer *et al.* 1997), up-regulating the anti-apoptotic genes (Fu *et al.* 2014) or by defending the oxidative damage to M-II oocyte spindle (Banerjee *et al.* 2012), therefore, indicating the positive influence of melatonin when added in combination with growth factors on the follicular development as was reported earlier (Wang *et al.* 2013; He *et al.* 2016).

From this study we observed that melatonin supplementation at an optimum dose of 100pM significantly improved the *in vitro* development of sheep PFs' and the yield of fertilizable oocytes from cultured PFs'. Concomitant supplementation of melatonin in the standard medium supported better growth of cultured preantral follicles. Therefore, it is concluded that melatonin (100pM) acts as survival factor for *in vitro* development of preantral follicles in sheep.

CONCLUSION

From this study we observed that melatonin supplementation at an optimum dose of 100pM significantly improved the *in vitro* development of sheep PFs' and the yield of fertilizable oocytes from cultured PFs'. Concomitant supplementation of melatonin in the standard medium supported better growth of cultured preantral follicles. Therefore, it is concluded that melatonin (100pM) acts as survival factor for *in vitro* development of preantral follicles in sheep.

ACKNOWLEDGEMENT

The authors thank Sri Venkateswara Veterinary University (S.V.V.U), Tirupati for providing the chemicals and laboratory facilities to conduct this work.

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