EFFECT OF MELATONIN ON NUCLEAR MATURATION OF CAPRINE OOCYTES

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ABSTRACT

Melatonin at the concentrations of 0, 5, 10, 20, 30, 40, and 50 ng/ml was used in maturation medium to assess the impact on *in vitro* maturation of caprine oocytes. The oocytes from slaughter house derived ovaries were subjected to *in vitro* maturation at 38.5°C with 5%CO_2. After culture for 27h, over 90% COCs had full cumulus cell expansion. The degree of cumulus cell expansion remained similar ($p>0.05$) on increasing melatonin concentration, however, oocytes incubated in 30 and 50 ng/ml melatonin containing maturation media for 27 h, result in 80% and 18.9% nuclear maturation rate, respectively, which were different ($p<0.05$) from control (45.3%). The nuclear maturation rate decreased ($p<0.05$) when melatonin concentration was increased from 30 to 40 and 50 ng/ml (80% *vs.* 28.3% and 18.9%, respectively). In conclusion, melatonin improved nuclear maturation of caprine oocytes at 30 ng/ml, whereas a high concentration of melatonin may affect caprine oocytes meiotic maturation at metaphase-II stage and can be toxic for caprine oocytes.

Keywords: Caprine, *in-vitro* maturation, Melatonin, Metaphase II, Oocytes.

INTRODUCTION

The manipulation of gametes and embryo increases the risk of exposure of these cells to high levels of reactive oxygen species (ROS) (Agrawal *et al*., 2006). Increased oxidative stress damages mitochondria and consequently impairs ATP production, and hampers meiotic and mitotic spindles formation in growing oocyte. It also destroys oocyte cell membrane lipids and DNA and progresses apoptosis quickly to inhibit fertilization (Kowaltowski *et al*., 1999).

Oxidative stress can be decreased by the presence of an antioxidant or radical scavenger in *in vitro* culture medium. Melatonin (MT; N-acetyl-5-methoxytryptamine) was successfully tested for promoting *in vitro* embryo development in many species including bovine (Manjunatha *et al*., 2009) and porcine (Kang *et al*., 2008). However, the effect of melatonin in maturation media for *in vitro* maturation of caprine oocytes has not been evaluated. Therefore, the present study was undertaken to investigate the optimum concentration of melatonin required for *in vitro* maturation of caprine oocytes.

MATERIALS AND METHODS

During spring season (February - April), goat ovaries (n=184) were collected from a local abattoir and were transported within 4h to laboratory in warm saline (35-37^{0}C), containing100 IU penicillin-G and 100 µg streptomycin sulfate per ml. Oocytes were retrieved by slicing goat ovaries and the recovered oocytes were graded (Kharche *et al.,* 2008).

Selected cumulus oocyte complexes (COCs) were washed two or three times in Oocyte Holding Medium (OHM) (TCM-199 medium, EGS 10%, Sodium Pyruvate 0.25 mM, Gentamicin 50 µg/ml, L-glutamine 100 µg/ml, BSA 3 mg/ml) and subsequently 8 to 10 times in 50-100 µl drops of oocyte maturation media supplemented with TCM-199 with 10% FBS, 10% follicular fluid, sodium pyruvate 0.25 mM, L-glutamine 100 µg/ml, LH 10µg/ml, FSH 5 µg/ml, estradiol-17β 1

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µg/ml, EGF 10ng/ml, BSA 3 mg/ml and gentamicin 50 µg/ml and allowed for maturation in 50 µl droplets of maturation media covered with sterile mineral oil for 27h in humidified atmosphere of 5% CO_2 at 38.5^{0}C in CO_2 incubator.

Matured oocytes (Fig. 1) were randomly divided into different treatment groups of maturation media on the basis of concentrations of melatonin added viz. group 1 (control, n=172), group 2 (5 ng/ml, n=120), group 3 (10 ng/ml, n=105), group 4 (20 ng/ml, n=125), group 5 (30 ng/ml, n=105), group 6 (40 ng/ml, n=113) and group 7 (50 ng/ml, n=111).

After 27 h maturation, oocytes were stripped off their cumulus cells by gentle pipetting for 1 min in 0.1% hyaluronidase enzyme. Denuded oocytes were selected and washed in PBS (1X) followed by fixation with Para-formaldehyde for 10 min. Oocytes were stained with Hoechst33342 dye (1µL/mL dissolved in DMSO was stored at 2–6° C, protected from light) for 30 min in dark. Therafter, oocytes were washed with 1X PBS and evaluated under an Inverted phase-contrast microscope. Nuclear stages were distinguished by the morphology of chromatin material (Hewitt *et al*., 1998; Yadav *et al*., 2013). Oocytes with second metaphase plate (two chromatin spot) and first polar body were classified as mature phase of second meiotic cell division (Fig. 2).

The maturation rates between different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 5% level of confidence.

RESULTS AND DISCUSSION

From slaughtered goat ovaries, by slicing technique, the oocyte recovery rate was 3.94. The nuclear stages of matured oocytes were distinguished by the morphology of chromatin material (Hewitt *et al*., 1998 and Yadav *et al*., 2013). Oocytes with first polar body or two chromatin spots were classified as mature phase of second meiotic cell division (MII).

Oocytes that show polar body or two chromatin spots were considered as matured oocytes (Fig. 1). The rate of oocytes maturation to MII stage was higher ($p<0.05$) with 30 ng/ml MT (80%), 20 ng/ml MT (68%), and 10 ng/ml MT (55.2%) as compared to control (45.3%, Table 1), possibly due to antioxidant role of melatonin. However, the oocyte maturation rate lowered in 40 ng/ml MT (28.3%) and 50 ng/ml MT (18.9%, Table 1). This could be due to toxic effect of melatonin at higher concentration, thus leading to lowering maturation rate and resulting in degeneration of oocytes (Tamura *et al*., 2009).

Melatonin (N-acetyl-5-methoxytryptamine) directly destroys free radicals, indirectly stimulates antioxidant enzymes and inhibits peroxidation enzymes such as nitric oxide synthetase (Galano *et al*., 2011). Melatonin accelerates the action of maturation-inducing hormone on maturation-promoting factor and germinal vesicle breakdown of oocytes (Chattoraj *et al*., 2005). The

Table 1: Nuclear maturation rate of caprine oocytes at different Melatonin concentrations

Melatonin, ng/ml	Oocytes, n	Matured oocytes, n	Maturation, %
0	172	78^{a,c}	45.4±0.4
5	120	56^{a,c}	46.5±0.2
10	105	58^{a,b}	55.1±0.3
20	125	86^{a,b}	68.6±0.1
30	105	84^{b}	80.2±0.1
40	113	32^{c}	28.2±0.3
50	111	22^{c}	19.5±0.1

$^{a,b}p<0.05$

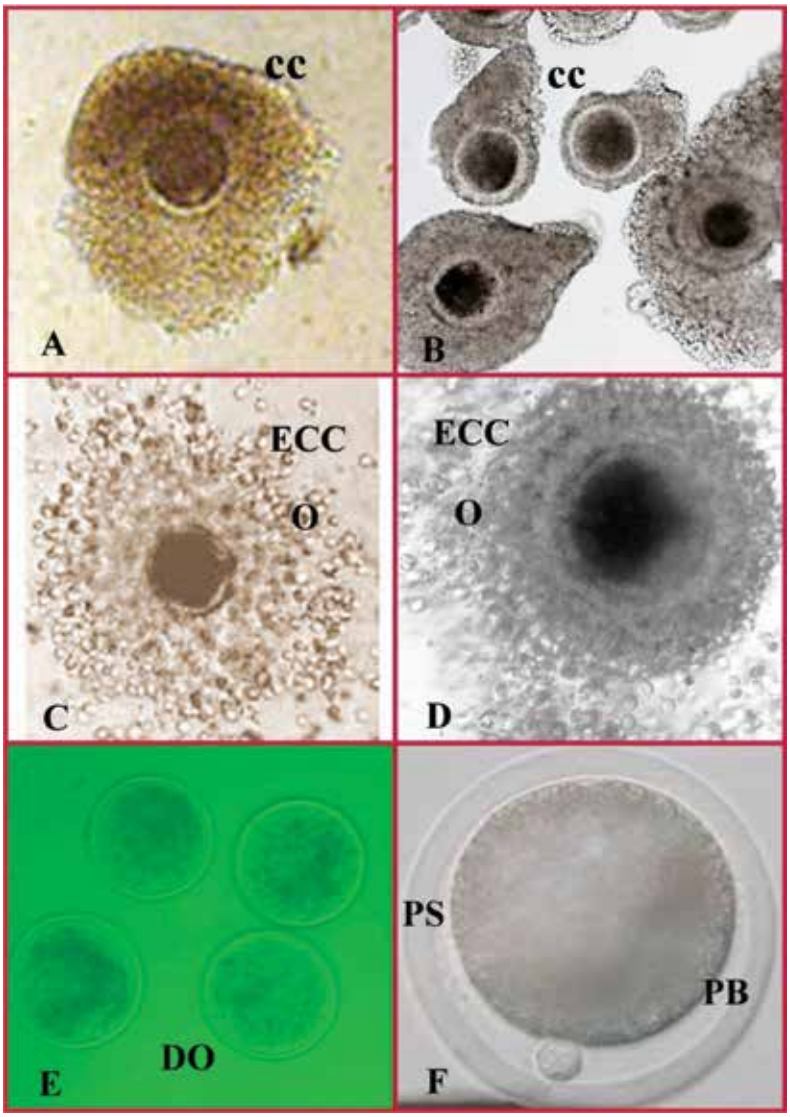

Fig. 1: Morphological maturation of caprine oocytes. A, B - Immature oocytes with compact cumulus cells (cc); C, D - Oocytes (O) with expanded cumulus cells (ECC) after *in vitro* maturation; E - Denuded oocytes (DO) after *in vitro* maturation; F - Matured oocytes with extruded polar body (PB) and peri-vitelline space (PS)

present study demonstrates that melatonin has a powerful antioxidative effect during IVM of bovine oocytes. In addition, the melatonin receptors were identified in granulosa cells, which suggest another possibility by which melatonin may take part in oocyte maturation.

The effect of different doses of melatonin on degree of nuclear maturation of oocytes investigated the optimal concentration for IVM of oocytes. The nuclear stages were identified as germinal vesicle stage (GV), germinal vesicle breakdown stage (GVBD), metaphase I stage (M I) and metaphase II stage (M II) with extruded polar body oocytes and Metaphase II plates were counted as mature (Fig. 2). The chromosomes in polar bodies with intact plasma membranes fluoresced blue. The observations from present study revealed

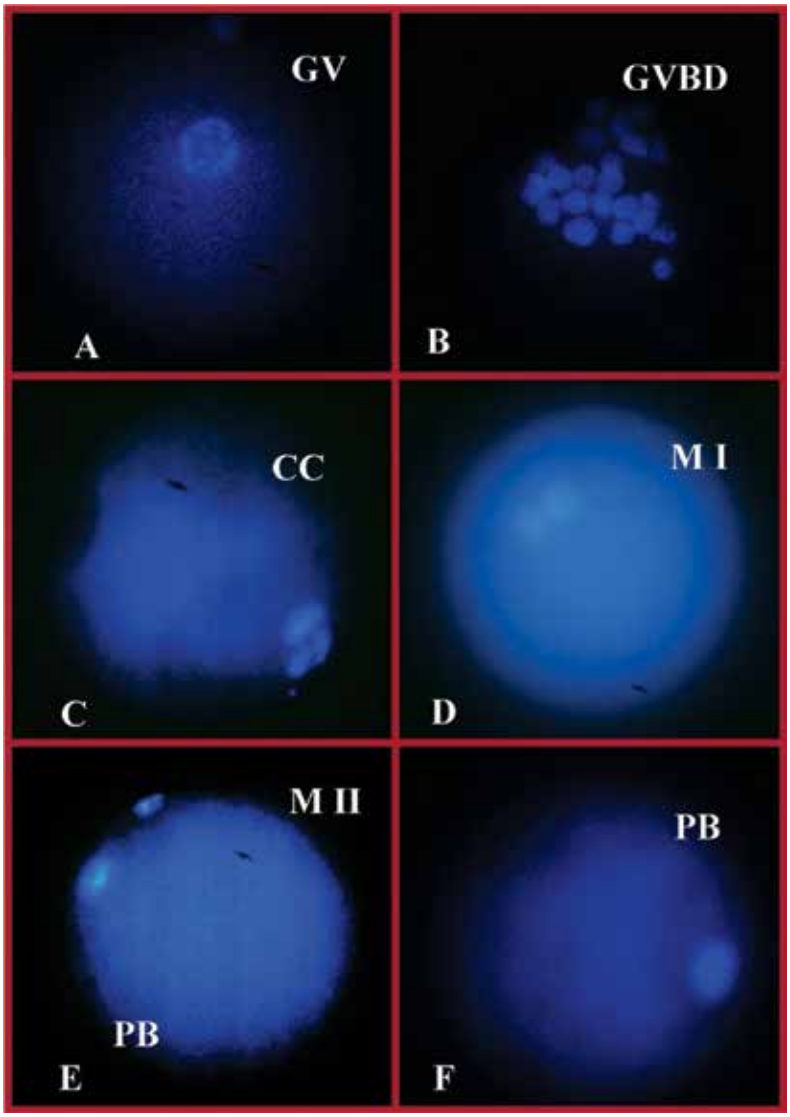

Fig. 2: Nuclear maturation of caprine oocytes. A - Oocyte showing germinal vesicle (GV) stage. Nuclear membrane is visible; B - Oocyte showing germinal vesicle break down stage (GVBD); C - Oocyte after GVBD with condensed chromatin mass (C); D - Oocyte with chromosomes arranged in equatorial plane at metaphase I (M I Stage); E, F - Oocytes showing metaphase II (M II stage) and extruded polar body (PB) under Hoechst staining (40X)

that all polar bodies had a sharply defined, smooth membrane and clear cytoplasm with chromosomes as scattered, stretched, or adherent to each other (Fig. 2).

In fact, the high concentrations of melatonin in follicular fluid (Brzezinski *et al.,* 1987), and the presence of receptors in granulose cells (Na *et al*., 2005), suggest that melatonin might be important to ovarian functions. The addition of melatonin to culture medium may increase the cumulus expansion and *in vitro* maturation of oocytes. The cumulus cells are known to play a crucial role during oocyte maturation. For acquiring developmental competence by oocyte *in vitro,* the cumulus cells during maturation are essential (Gordon, 2003). It was reported that 1 µM melatonin

reduces cumulus cells apoptosis by activating its receptors on cumulus cells (Na *et al*., 2005).

Thus, melatonin improved the nuclear maturation of caprine oocytes at 30 ng/ml, whereas a high concentration of affected *caprine* oocytes meiotic maturation at metaphase-II stage and can be toxic for caprine oocytes.

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