

## Effect of melatonin on maturation capacity and fertilization of Nili-Ravi buffalo (*Bubalus bubalis*) oocytes

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### Abstract

This study evaluated the effect of melatonin supplementation of *in vitro* maturation media on *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) rate of buffalo oocytes. Cumulus oocytes complexes (COCs) were aspirated from follicles of 2-8 mm diameter. In experiment I, COCs were matured in IVM medium supplemented with 0 (control), 250, 500, and 1000  $\mu$ M melatonin for 22-24 hours in CO<sub>2</sub> incubator at 38.5°C with 5% CO<sub>2</sub> and at 95% relative humidity. The maturation rate did not differ in media supplemented with melatonin at 250  $\mu$ M, 500  $\mu$ M, 1000  $\mu$ M and control (0  $\mu$ M). In experiment II, the matured oocytes were fertilized in 50  $\mu$ l droplets of Tyrode's Albumin Lactate Pyruvate (TALP) medium having 10  $\mu$ g/ml heparin for sperm (2 million/ml) capacitation. The fertilization droplets were then kept for incubation at 5% CO<sub>2</sub>, 39°C and at 95% relative humidity for 18 hours. The fertilization rate was assessed by sperm penetration and pronuclear formation. Fertilization rate was improved when maturation medium was supplemented with 250  $\mu$ M melatonin compared to control. In conclusion, melatonin supplementation to serum free maturation media at 250  $\mu$ M improved the fertilization rate of buffalo oocytes.

**Keywords:** Buffalo, *In vitro* fertilization, *In vitro* maturation, Melatonin.

### Introduction

Buffalo is important for ecologically disadvantaged agricultural systems as it not only provides meat, milk and working power but is also essential as a livestock source. Due to low maintenance requirements and good ability of feed conversion, buffaloes are considered ideal for low input systems and for the low cost production systems (Zicarelli, 1994). In spite of these qualities, the production potential of our dairy buffalo is low compared to dairy cattle in developed countries. Therefore consistent efforts are being made to improve the genetic potential of buffalo through assisted reproductive technologies. Artificial insemination that utilizes the superior male germplasm has been developed and is in use to some extent. Embryo transfer that utilizes superior male and female germplasm simultaneously is at the verge of experimentation in Pakistan. The efficiency of *in vitro* embryo production (IVEP) is lower in buffalo compared to cattle (Nandi *et al.*, 2002). The low number of primordial and antral follicles as well as high incidence of follicular atresia is the major impediment for the *in vitro* embryo production in this species (Palta *et al.*, 1998).

When cultured *in vitro*, oocytes are exposed to light, increased oxygen concentrations, increased or decreased concentrations of metabolites and substrates during handling of oocytes that causes oxidative stress (Agarwal *et al.*, 2006). Naturally, free radical-scavenging antioxidants exist within the follicular and oviductal fluid that is able to protect the oocytes against

oxidative stress (Wang *et al.*, 2002), however, this system becomes insufficient under *in vitro* conditions. Melatonin and its metabolites have the ability to scavenge directly the free radicals and indirectly to act as powerful antioxidant (Adriaens *et al.*, 2006; Kang *et al.*, 2009). It has been reported that melatonin directly protects the oocytes of human and mouse from oxidative stress (Tamura *et al.*, 2008). In bovine oocytes, melatonin stimulated the re-initiation of meiosis but it was unable to complete the meiosis or cleavage of oocytes after their maturation and fertilization in the laboratory (Sirotkin and Schaeffer, 1997). Present study was designed to assess the role of melatonin in *in vitro* maturation media on *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of buffalo oocytes.

### Materials and Methods

This study was conducted at Physiology Laboratory, Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi.

#### Reagents and chemicals

The reagents and chemicals used in the study were purchased from Sigma (St. Louis MO, USA), or mentioned when purchased from other source.

#### Collection of ovaries

Nili-Ravi buffalo ovaries were collected immediately after slaughtering from local slaughterhouse at Sihala (Islamabad), and transported to laboratory within two hours in a thermos having sterilized saline solution held at 37 °C (Mehmood, 2007). In the laboratory, ovaries were washed with 70% ethanol for 30 seconds

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followed by three times rinse in saline solution (Jamil, 2007).

#### **Retrieval of oocytes**

A 10 ml syringe attached to a needle (18 gauge) was used to retrieve cumulus oocyte complexes from follicles having diameter of 2-8 mm. The follicular fluid was collected in a conical tube and kept for 10-15 minutes. After discarding the supernatant, the sediment was collected in 60 mm petri dish and oocytes were searched under stereo microscope.

#### **Classification of oocytes**

The cumulus oocytes complexes were graded as: A Grade: having evenly granulated homogenous ooplasm with cumulus cells of three or more compact layers, B Grade: having homogenous ooplasm with two to three layers of cumulus cells, C Grade: having irregular ooplasm with less compact cumulus cells and D Grade: having irregular dark ooplasm and highly expanded cumulus cells (Singhal et al., 2009).

#### **Maturation of oocytes**

Total number of buffalo ovaries used in the present study was 1179 and the numbers of oocytes recovered were 1300. Only grade A (n= 315) and B (n=290) oocytes were used for INM. The oocytes were washed twice with oocytes wash media and once with IVM media (pH 7.3-7.4). The oocytes were placed in IVM medium (Jamil et al., 2007) with some modifications (MM; TCM-199 supplemented with BSA 6 mg/mL, 10 IU/mL LH, 0.5 ug/mL FSH, 1 ug/mL estradiol-17 $\beta$  and 50 ug/mL gentamicin) alone or with melatonin supplemented at 250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M in four experimental groups covered with mineral oil. The maturation dishes containing oocytes were placed for 22-24 hours in an incubator with 5% CO<sub>2</sub>, at 39 °C and at humidity of about 95%.

#### **Sperm preparation**

Swim up technique was used to separate the most motile spermatozoa in medium known as Tyrode's albumin lactate pyruvate (Sperm TALP) (Jamil et al., 2007). Briefly, 3 mL of TALP medium (pH 7.3-7.4) was taken in each of the four 15 ml tubes and incubated for two hours in an incubator at 39°C temperature and 5% CO<sub>2</sub>. Cryopreserved buffalo semen was thawed at 37°C for 30 seconds. About 70  $\mu$ l of thawed semen was transferred into four 15 ml tubes containing sperm TALP, placed at an angle of 45° and incubated at 39°C, 5% CO<sub>2</sub> and 95% humidity. The supernatant containing the most motile spermatozoa was transferred to a 15 ml falcon tube for centrifugation at 1600 rpm for 10 minutes. The sperm pellet was suspended in fertilization TALP having 10 $\mu$ g/mL heparin for capacitation. Neubauer hemocytometer was used for measuring sperm concentration (Hafez and Hafez, 2000) and sperm concentration was adjusted to 1 $\times$ 10<sup>6</sup>/mL by adding fertilization media.

#### **In vitro fertilization**

Following maturation, oocytes were washed three times in the fertilization TALP and then 5-10 oocytes

were incubated in 50  $\mu$ l droplets of fertilization TALP (mTALP) having sperm under mineral oil at 5% CO<sub>2</sub>, 95% humidity and 39 °C for 18 hours.

#### **Evaluation of in vitro maturation**

##### *Cumulus cell expansion*

After 24 hour of incubation, the degree of cumulus expansion was assessed using stereo microscope as; not expanded oocytes without loosened, partially expanded with slightly loosened or fully expanded oocytes with cumulus cells completely loosened.

##### *Stage of nuclear maturation*

After IVM, oocytes were vortexed in 1 ml M-199 containing 300  $\mu$ l/mL hyaluronidase for 2 minutes to completely denude the oocytes. The denuded oocytes were washed with simple maturation media, placed on grease-free slide and cover slipped with 4 droplets of Vaseline/Paraffin (40:1) mixture. Oocytes were slightly compressed and fixative (acetic acid and ethanol in the ratio of 1:3 v/v) was used for fixing oocytes for 5 minutes. Acetoorcein stain (1% orcein in 45% acetic acid) was used for staining of oocytes. After 3-5 minutes, excess stain was removed from oocytes by a de-staining solution composed of acetic acid: distilled water: glycerol (1:3:1) in the same manner as staining of oocytes. Phase contrast microscope (400X) was used to determine the stage of maturation (Hewitt et al., 1998). Oocytes were classified at germinal vesicle (GV; prophase I having a prominent nucleolus), germinal vesicle breakdown (GVBD; condensation of chromosomes by resolution of nuclear membrane), metaphase I (M-I; arrangement of chromosomes at equator but without a polar body) and metaphase II (M-II; when polar body was extruded).

##### *Assessment of in vitro fertilization*

After 18 h of insemination, oocytes were stained as described earlier. Oocytes with penetrating head, with male pronucleus (MPN) and female pro-nucleus (FPN) were considered as normal fertilized. Oocytes with three or more pro-nuclei were considered as polyspermic.

#### **Statistical analysis**

The data on maturation rate of oocytes and IVF rate of *in vitro* matured oocytes were analyzed by chi square analysis. Statistically significant confidence interval was taken as P<0.05.

## **Results**

#### **Recovery of oocytes**

Oocytes were classified into four different grades on the basis of homogenous ooplasm and the compactness of the cumulus cells as shown in the Fig. 1. Data on the recovery of different grade oocytes from the ovaries is given in the Table 1. Total oocytes recovered from buffalo ovaries (n=1179) were 1300 in number which were retrieved from follicles of 2-8 mm by aspiration method. Out of these oocytes grade C oocytes were higher (28.07%) followed by grade D (25.38%), grade A (24.23%) and grade B (22.30%).

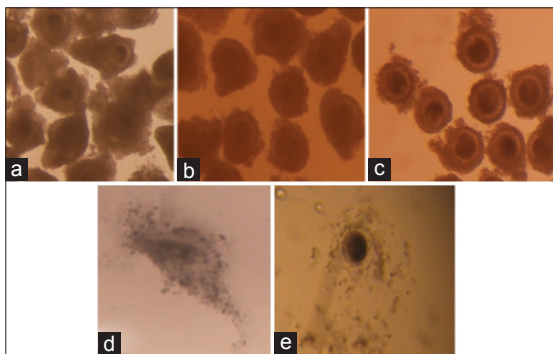
### Oocytes maturation in IVM media supplemented with melatonin

#### Expansion of Cumulus Cells

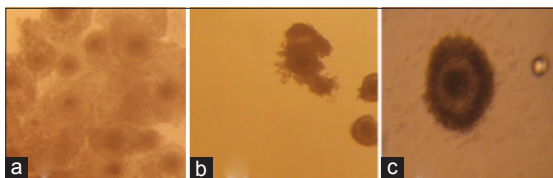
The data on degree of cumulus expansion of buffalo oocytes with the supplementation of melatonin in IVM media are given in the Table 2 and Fig. 2 as well. Fully expanded oocytes were 33.04, 42.85, 38.88 and 32.50% in control, 250  $\mu$ M, 500  $\mu$ M, and 1000  $\mu$ M melatonin supplemented maturation media, respectively.

#### Degree of maturation of oocytes

The data on the number of oocytes at different stages of maturation after 24 hours of incubation are given in the Table 3 and Fig. 3. The oocytes that remained at GV stage were 5.12% and 3.03% in control and 500  $\mu$ M melatonin supplemented media. While no oocyte was found at GV stage in the media supplemented with 250  $\mu$ M and 1000  $\mu$ M of melatonin. The oocytes reaching GVBD were 12.82% in control, while 6.06%, 9.09% and 6.25% in media supplemented with 250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M melatonin. The oocytes at M-I stage were 30.76%, 24.24%, 24.24% and 28.12% in control, 250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M melatonin supplemented media, respectively. The MII stage oocytes were recorded as, 51.28% 69.69%, 63.63% and 65.62% in control, 250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M melatonin supplemented media, respectively. Oocytes



**Fig. 1.** Recovery of different grades oocytes from buffalo ovaries by aspiration. (a) Grade A oocyte with cumulus cells of 3-4 layers. (b) Grade B oocyte with cumulus cells of 2-3 layers. (c) Grade C oocytes cumulus cells maximum 1 layer. (d) Grade D oocyte highly denuded (e) or denuded (f). (400x by stereo microscope).



**Fig. 2.** Degree of expansion of oocytes complexes after 24 hours of IVM. (a) Fully expanded cumulus layer. (b) Partially expanded cumulus layer. (c) Not expanded cumulus layer. (400x by stereo microscope).

at M-II were considered to be matured, the percentage of which in the media supplemented with different concentrations of melatonin was found slightly higher compared to control, however the difference remained non-significant ( $P>0.05$ ).

#### Fertilization of oocytes (IVF)

The data on the effect of IVM media supplemented with different concentrations of melatonin on fertilization of buffalo oocytes after 18 hours of insemination are given in the Table 4 and Fig. 4. The oocytes that were penetrated remained 2.3% in control and 10.25% when the oocytes were matured in 1000  $\mu$ M melatonin supplemented IVM media. Oocytes with 2PN were recorded as 25.58%, 46.15%, 43.58%, and 23.07% in control, 250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M melatonin supplemented groups. A small number of polyspermic oocytes (10.25%) were found when the oocytes were matured in media supplemented with 1000  $\mu$ M melatonin. The number of oocytes that remained unidentified was 2.3%, 10.25%, 15.4% and 15.38%, in control, 500  $\mu$ M, 250  $\mu$ M and 1000  $\mu$ M melatonin supplemented media.

**Table 1.** Frequency distribution of different grades of Oocytes recovered from buffalo ovaries (n=1179) by aspiration method.

| Oocytes               | Recovery rate |               |
|-----------------------|---------------|---------------|
|                       | Total         | Per ovary (%) |
| Oocytes recovered     | 1300          | 1.1           |
| Usable oocytes (A, B) | 605           | 0.51          |
| Grade A (%)           | 315 (24.2)    | 0.26          |
| Grade B (%)           | 290 (22.3)    | 0.25          |
| Grade C (%)           | 365 (28.1)    | 0.31          |
| Grade D (%)           | 330 (25.4)    | 0.28          |

**Table 2.** Effect of supplementation of melatonin in media for IVM on degree of cumulus expansion of buffalo oocytes after 24 hours of maturation.

| IVM Media                 | No. of oocytes | Degree of cumulus expansion n (%) |                    |                |
|---------------------------|----------------|-----------------------------------|--------------------|----------------|
|                           |                | Not expanded                      | Partially expanded | Fully expanded |
| Maturation media (MM)     | 115            | 34 (29.6)                         | 43 (37.4)          | 38 (33.0)      |
| Mm+250 $\mu$ M melatonin  | 112            | 32 (28.6)                         | 32 (28.6)          | 48 (42.8)      |
| Mm+500 $\mu$ M melatonin  | 108            | 31 (28.7)                         | 35 (32.4)          | 42 (38.9)      |
| Mm+1000 $\mu$ M melatonin | 120            | 42 (35.0)                         | 39 (32.5)          | 39 (32.5)      |

Chi-square analysis,  $p>0.05$

**Table 3.** Effect of supplementation of melatonin in media for IVM on nuclear maturation of buffalo oocytes after 24 hours of in vitro maturation.

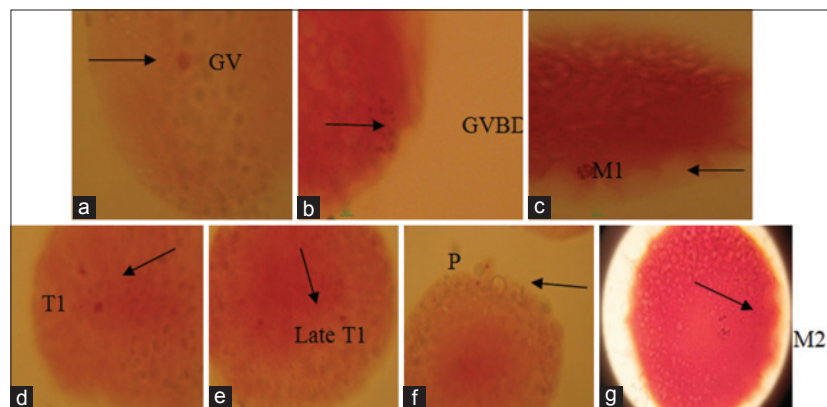
| Treatments                | No. of Oocytes | n (%)   |          |           |           |
|---------------------------|----------------|---------|----------|-----------|-----------|
|                           |                | GV      | GVBD     | M1        | M2        |
| Maturation media (MM)     | 39             | 2 (5.1) | 5 (12.8) | 12 (30.8) | 20 (51.3) |
| MM+250 $\mu$ M melatonin  | 33             |         | 2 (6.1)  | 8 (24.2)  | 23 (69.7) |
| MM+500 $\mu$ M melatonin  | 33             | 1 (3.0) | 3 (9.1)  | 8 (24.2)  | 21 (63.7) |
| MM+1000 $\mu$ M melatonin | 32             |         | 2 (6.3)  | 9 (28.1)  | 21 (65.6) |

Chi-square analysis,  $p > 0.05$

**Table 4.** Effect of supplementation of melatonin in media for IVM on in vitro fertilization of buffalo oocytes after 18 hours of insemination.

| Treatments                | No. of oocytes |                        |           |            |             |                |                |
|---------------------------|----------------|------------------------|-----------|------------|-------------|----------------|----------------|
|                           | Inseminated    | Fertilized             | With 2PN  | Penetrated | Polyspermic | Not fertilized | Not identified |
| Maturation media (MM)     | 43             | 12 (27.9) <sup>b</sup> | 11 (25.6) | 1 (2.3)    |             | 30 (69.8)      | 1 (2.3)        |
| MM+250 $\mu$ M melatonin  | 39             | 18 (46.2) <sup>a</sup> | 18 (46.2) |            |             | 15 (38.5)      | 6 (15.4)       |
| MM+500 $\mu$ M melatonin  | 39             | 17 (43.6) <sup>b</sup> | 17 (43.6) |            |             | 20 (51.3)      | 4 (10.3)       |
| MM+1000 $\mu$ M melatonin | 39             | 17 (43.6) <sup>b</sup> | 9 (23.1)  | 4 (10.3)   | 4 (10.3)    | 22 (56.4)      | 6 (15.4)       |

MM: Maturation media; PN: Pro-nucleus; Chi-square analysis,  $p < 0.05$



**Fig. 3.** Different stages of nuclear maturation after 24 hours of IVM. (a) Germinal vesicle (GV), showing nucleolus. (b) Germinal vesicle breakdown (GVBD), showing condensation of chromosomes. (c) Metaphase I (MI), showing chromosomes at meiotic plate without any polar body. (d) Telophase (T1) stage. (e) Late Telophase (T1). (f) Metaphase II (M2), stage showing extrusion of first polar body. (g) Metaphase II (M2). (400x by phase contrast microscopy).

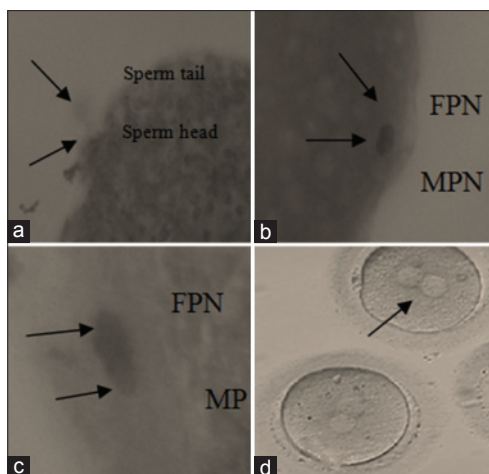
The overall percentage of fertilized oocytes in control and in the media supplemented with 250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M were 27.90%, 46.15%, 43.58% and 43.58% respectively. The percentage of fertilized oocytes in the media supplemented with 500  $\mu$ M and 1000  $\mu$ M melatonin did not differ significantly from the control (MM+BSA). However, percentage of fertilized oocytes was higher ( $P < 0.05$ ) when the oocytes were matured in media supplemented with 250  $\mu$ M melatonin compared to control.

#### Discussion

Maturation involves important events which an oocyte needs to complete for successful fertilization and early

embryogenesis. Appropriate maturation is the basis for implantation, initiation of pregnancy, and fetal development (Brevini and Gandolfi, 2001; Sirard *et al.*, 2006). Generally, maturation involves accumulation of mRNA, proteins, substrates, and nutrients that are required to achieve oocyte's developmental competence that fosters embryonic developmental competence (Brevini and Gandolfi, 2001; Krisher, 2004; Sirard *et al.*, 2006).

The oocytes are more exposed to light, air and chemicals during *in vitro* handling that is responsible for generation of reactive oxygen species (ROS). Buffalo oocytes were found much sensitive to stress caused by



**Fig. 4.** Different stages of *in vitro* fertilization (IVF) of buffalo oocytes after 18 hours of insemination. (a) Penetrated oocytes showing sperm head and tail. (b&c) Fertilized oocytes showing fused male (MPN) and female pro-nucleus (FPN). (d) Fertilized oocyte with two nuclei. (400x by phase contrast microscopy).

reactive oxygen species during culturing in laboratory (Boni *et al.*, 1992). The oocyte developmental competence was reported to be improved by increasing the antioxidant capacity of oocytes during IVM, as the high lipid content makes buffalo oocytes/embryos sensitive to oxidative damages (Boni *et al.*, 1992).

Melatonin has the ability to scavenge oxygen radical, and is effective in reducing apoptosis in different cell types (Allegra *et al.*, 2003; Juknat *et al.*, 2005). Melatonin reduces oxidative stress of ovarian follicles and helps in protection of oocytes from free radical damage (Takasaki *et al.*, 2003; Tamura *et al.*, 2008). Melatonin receptors have been reported on cumulus cells and when supplemented in maturation media, it reduced apoptosis of cumulus cells in mouse (Na *et al.*, 2005). Further, improvement in maturation and cleavage rates with the supplementation of melatonin in IVM and IVF medium was reported in mice (Na *et al.*, 2005), bovine (Dimitriadis *et al.*, 2005) and humans (Parka *et al.*, 2006). Similarly, positive effect of melatonin was reported during *in vitro* embryo development in mouse (Ishizuka *et al.*, 2000), pig (Rodriguez *et al.*, 2007, Kang *et al.*, 2009, Shi *et al.*, 2009) and buffalo (Manjunatha *et al.*, 2009).

The overall recovery rate of oocytes in our study was lower as the study was conducted in the low breeding season that affects the overall production of progesterone and estradiol required for follicular growth and development. In present study, the percentage of fully expanded COCs decreased progressively with increasing melatonin concentration but difference in the degree of cumulus expansion between different concentrations of melatonin and control was not

significant ( $P>0.05$ ). The dose dependent effect of melatonin on cumulus cells expansion has previously been reported in mouse (Adriaens *et al.*, 2006).

The rate of nuclear maturation did not differ significantly with melatonin supplementation (250  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M) of IVM and control (0  $\mu$ M) in the present study ( $P>0.05$ ) as reported earlier that higher concentrations of melatonin might have negative effect on survival of follicles leading to lower rates of maturation (Adriaens *et al.*, 2006). In contrast to our study, useful effects of melatonin have been reported during porcine IVM (Kang *et al.*, 2009). Similarly, increase in the maturation rate and transformable embryo yield was reported earlier in buffalo when melatonin was supplemented in IVM media (Manjunatha *et al.*, 2009). Furthermore, as the melatonin receptors are present in bovine cumulus cells and its lower concentrations (0.1 pM to 1000 nM) may positively affect cytoplasmic and nuclear maturation (Raey *et al.*, 2011), however, it was not observed in present study.

In present study, the IVF rate of oocytes was independent of maturation rate and was significantly improved in the group treated with 250  $\mu$ M of melatonin. The beneficial effects of melatonin on *in vitro* embryo development have already been reported in mouse (Gao *et al.*, 2012) and in buffalo (Zhang *et al.*, 1995). However, in bovine, melatonin supplementation of IVM media did not improve the cleavage and blastocyst rates (Tsantarliotou *et al.*, 2007). The supplementation of melatonin in IVM and IVF medium simultaneously, improved maturation and cleavage rates in mice (Na *et al.*, 2005), bovine (Dimitriadis *et al.*, 2005) and humans (Parka *et al.*, 2006). This study showed that increasing melatonin concentrations beyond 250  $\mu$ M does not have any significant effect on maturation rates of buffalo oocytes. Melatonin at higher concentrations may be toxic and may result in cell injury and lower blastocyst rates due to its toxicity (Rodriguez *et al.*, 2007). So, it is concluded that addition of melatonin at 250  $\mu$ M improved the fertilization rates of buffalo oocytes, although it did not show improvement IVM rate of buffalo oocytes.

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