**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 μM melatonin for 22-24 hours in CO₂ incubator at 38.5°C with 5% CO₂ and at 95% relative humidity. The maturation rate did not differ in media supplemented with melatonin at 250 μM, 500 μM, 1000 μM and control (0 μM). In experiment II, the matured oocytes were fertilized in 50 μl droplets of Tyrode’s Albumin Lactate Pyruvate (TALP) medium having 10 ug/ml heparin for sperm (2 million/ml) capacitation. The fertilization droplets were then kept for incubation at 5% CO₂, 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 μM melatonin compared to control. In conclusion, melatonin supplementation to serum free maturation media at 250 μM 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 (IVP) 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 artesia 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 thermost 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 guage) 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-17β and 50 ug/mL gentamicin) alone or with melatonin supplemented at 250 μM, 500 μM and 1000 μM in four experimental groups covered with mineral oil. The maturation dishes containing oocytes were placed for 22-24 hours in an incubator with 5% CO₂, 95% humidity and 39 °C for 18 hours.

**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% CO₂.

**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**

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 μM, 500 μM, and 1000 μM melatonin supplemented maturation media, respectively. While no oocyte was found at GV stage in the media supplemented with 250 μM and 1000 μM of melatonin. The oocytes reaching GVBD were 12.82% in control, while 9.09% and 6.25% in media supplemented with 250 μM, 500 μM and 1000 μM melatonin. The oocytes at M-I stage were 30.76%, 24.24%, 24.24% and 28.12% in control, 250 μM, 500 μM and 1000 μM melatonin supplemented media, respectively. The MII stage oocytes were recorded as, 51.28% 69.69%, 63.63% and 65.62% in control, 250 μM, 500 μM and 1000 μM melatonin supplemented media, respectively. Oocytes 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).

**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 μM melatonin supplemented media. While no oocyte was found at GV stage in the media supplemented with 250 μM and 1000 μM of melatonin. The oocytes that were penetrated remained 2.3% in control and 10.25% when the oocytes were matured in 1000 μM melatonin supplemented IVM media. Oocytes with 2PN were recorded as 25.58%, 46.15%, 43.58%, and 23.07% in control, 250 μM, 500 μM and 1000 μM melatonin supplemented groups. A small number of polyspermic oocytes (10.25%) were found when the oocytes were matured in media supplemented with 1000 μM melatonin. The number of oocytes that remained unidentified was 2.3%, 10.25%, 15.4% and 15.38%, in control, 500 μM, 250 μM and 1000 μM 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 μM melatonin | 112 | 32 (28.6) | 48 (42.8) |
| Mm+500 μM melatonin | 108 | 31 (28.7) | 42 (38.9) |
| Mm+1000 μM melatonin | 120 | 42 (35.0) | 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 | GV   | GVBD | M1   | M2   |
|-----------------------------|----------------|------|------|------|------|
| Maturation media (MM)       | 39             | 2 (5.1) | 5 (12.8) | 12 (30.8) | 20 (51.3) |
| MM+250 μM melatonin        | 33             | 2 (6.1) | 8 (24.2) | 23 (69.7) |
| MM+500 μM melatonin        | 33             | 1 (3.0) | 3 (9.1) | 8 (24.2) | 21 (63.7) |
| MM+1000 μM 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)^b| 11 (25.6) | 1 (2.3)  | 30 (69.8)  | 1 (2.3)     |
| MM+250 μM melatonin        | 39             | 18 (46.2)^a| 18 (46.2) |          | 15 (38.5)  | 6 (15.4)    |
| MM+500 μM melatonin        | 39             | 17 (43.6)^b| 17 (43.6) |          | 20 (51.3)  | 4 (10.3)    |
| MM+1000 μM melatonin       | 39             | 17 (43.6)^b| 9 (23.1)  | 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 2 (M2), stage showing extrusion of first polar body. (g) Metaphase 2. (400x by phase contrast microscopy).

The overall percentage of fertilized oocytes in control and in the media supplemented with 250 μM, 500 μM and 1000 μM were 27.90%, 46.15%, 43.58% and 43.58% respectively. The percentage of fertilized oocytes in the media supplemented with 500 μM and 1000 μM 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 μM 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
Melatonin reduces oxidative stress of ovarian follicles cell types (Allegra, M., Reiter, R.J., Tan, D.X., Gentile, C., Agarwal, A., Tamer, S., Mohammed, A.B., Jashoman, B. 2008). Melatonin has the ability to scavenge oxygen radical, sensitive to oxidative damages (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 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 (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 μM 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 μM improved the fertilization rates of buffalo oocytes, although it did not show improvement IVF rate of buffalo oocytes.

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