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Effect of melatonin on *in vitro* maturation of buffalo (*Bubalus bubalis)* oocytes

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Abstract
Objective: Nutrients and antioxidants in the medium of immature oocyte have a profound effect on maturation, fertilization and development of resulting embryos. In this study the effects of varying dose of melatonin as an antioxidant agent on maturation capacity were evaluated in immature oocytes of buffaloes.
Materials and Methods: In this experimental study, immature oocytes were harvested from ovaries of buffaloes slaughtered at abattoirs of Chennai Corporation. Oocytes were harvested from collected ovaries by using different techniques and within 30 minutes it was transferred to *in vitro* maturation medium containing varying doses of melatonin (10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}) and without melatonin (Control) for 22-24 hours. Maturation was monitored using an inverted microscope.
Results: Melatonin in the concentration of 10^{-6} M had the most effect on maturation of oocytes in *in vitro* maturation medium ($p<0.05$) when compared to the control. This study concludes that melatonin at the rate of 10^{-6} M in the serum free IVM plays an effective role in attaining the maximal maturation rate of buffalo oocytes.

Keywords: buffalo, *in vitro* maturation, melatonin, oocytes

1. Introduction
Buffalo is the mainstay of Indian dairy industry since it contributes over 55% of the milk production. Despite the importance of buffalo to the socioeconomic status, its population has been declining, partly due to poor reproductive performance. The low reproductive efficiency in female buffalo can be attributed to delayed puberty, higher age at calving, long postpartum anoestrus period, long calving interval, lack of overt sign of heat and low conception rate (Kumar and Anand, 2012) [22]. In addition, female buffaloes have a lower number of follicles in the ovary; poor super ovulatory response and high percentage of atretic follicles (Haldar and Prakash, 2007; Hufana-Duran et *al*., 2007) [13, 16].
Oocyte maturation is the first and most critical step towards successful *in vitro* embryo production buffalo embryos has been gaining attention for its research and commercial application ever since the birth of the first buffalo calf through *in-vitro* fertilization of buffalo oocytes (Totey *et al*.,1992). Although the quality of *in vitro* matured oocytes (IVM) is less than *in vivo* matured oocytes (Moor and Dai, 2001), it is a frequent technique carried out by *in vitro* fertilization (IVF) centres for augmenting more number of mature oocytes for IVF. The culture medium and selection of protein supplements and hormones for IVM plays an important role in the subsequent maturation rate and embryonic development following IVF (Bavister *et al*., 1992) [4].
In vitro handling and culture conditions causes oocytes and embryos to oxidative stress resulting from events such as exposure to light, elevated oxygen concentrations and unusual concentration of metabolites and substrates (Agrawal *et al*., 2006) [1].
Del Corso *et al*., 1994 found that *in vitro* mammalian embryo development is negatively affected by the increased oxidative stress occurring under culture conditions. The oxidative damage of cell components via reactive oxygen species interferes with proper cell function. Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anions, or hydroxyl radical, damage cell membranes and DNA play a role in apoptosis (Kitagawa *et al*., 2004; Agrawal *et al*., 2006) [1]. Therefore, it is important to protect oocytes from oxidative stress during *in vitro* maturation (IVM). Most mammalian cells possess efficient antioxidant systems such as catalase or superoxide dismutase, as well as thiol compounds that act as metabolic buffers which scavenge active oxygen species.

The protective role of free radical scavengers in maturation culture medium has been documented (Tsantarioutou *et al*., 2007, Kang *et al*., 2009, Manjunatha *et al.,* 2009) [37, 20, 25]. Melatonin acts as a potent scavenger of free radicals (Tan *et al*., 2007) [35] and a direct gonadal response to melatonin has also been reported, including stimulation of progesterone (Adriaens *et al*., 2006) [2] and esradiol synthesis (Sirotkin and Schaeffer, 1997) and improvement of oocyte quality and maturation (Chattorag *et al*., 2005; Kang *et al*., 2009) [20].

Melatonin (N-aceyl-5-methoxytryptamine), an indole derivative secreted rhythmically from the pineal gland, plays a major role in regulating the circadian clock in mammals (Reiter, 1991) [31]. Also this molecule has major effects on the reproductive system in mammals (Reiter, 1998; Sirotkin and Schaeffer, 1997) [32, 34]. More recent studies have demonstrated that beside its multiple actions on different physiological processes, melatonin as well as its metabolites act as indirect antioxidants and powerful direct scavengers of free radicals (Reiter, 1998) [32].

In contrast to the majority of other known radical scavengers, melatonin is multifunctional and universal (Hardeland 2005, Leon, 2005, Tomas-Zapico, 2005) [14, 24, 36]. It is soluble both in water and in lipids and hence acts as a hydrophilic and hydrophobic antioxidant. The beneficial effect of supplementing culture medium with melatonin had been reported in mouse (Ishizuki, 2000) [17] and bovine (Poleszczuk *et al*., 2007) [30] *in vitro* embryo development. It has been reported that exogenous melatonin has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM (Kang *et al*., 2008).

Melatonin has been successfully tested for promoting mouse embryo development *in vitro* (Ishizuka *et al*., 2000) [17]. It has also been reported as having no detrimental effects on mouse or rat embryo development during toxicity tests (Melhinny *et al.,* 1996) [26] performed either *in vitro* or *in vivo* (Jahnke *et al.,* 1999) [18]. Na *et al.* 2005 [29] have found that the addition of melatonin in the IVM medium significantly improved nuclear maturation of the GV mouse oocytes and the highest maturation rate were found in the group treated with 1.0μM melatonin. In the group treated with 1.0μM melatonin, melatonin receptor (Mel1a) mRNA was highly expressed in IVM oocytes and cumulus cells.

Several studies have shown that enriching the culture medium with melatonin can improve embryo development, but blastocyst rates can vary widely depending on the melatonin concentrations (Ishizuka *et al*., 2000; Danilova *et al*., 2004; Manjunatha *et al*., 2008) [17]. In addition, melatonin can increase blastocyst rates and blastocyst total cell number concomitant to a significant decrease of apoptotic nuclei rate in preimplantational parthenogenetic porcine embryos (Cui *et al*., 2008) [7]. Hence the present study is undertaken to find out the optimum dose of melatonin required for maturation of oocytes which attains full maturity.

Materials and Method

The present study was carried out in the Centralized Embryo Biotechnology Unit at Madhavaram and Molecular Biology Lab at Department of Animal Biotechnology, Chennai.

Reagents and chemicals

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

Collection of ovaries

Ovaries from sexually mature buffaloes (*Bubalus bubalis*) were collected irrespective of age, body condition, stage of estrus cycle and season from Perambur and Pallavaram Chennai corporation abattoir. In this study the ovaries were removed within 30 minutes of slaughter and washed in phosphate buffer saline (PBS) supplemented with 50μg/ml gentamicin sulphate to remove blood and extraneous material. The washed ovaries were transported at 37 ^{0}C in a thermos flask in the same media to the laboratory within 30 minutes.

Retrieval of Oocytes

Cumulus Oocyte complexes were retrieved from buffalo ovaries by aspiration and slicing techniques.

Retrieval of oocyte by aspiration techniques

COCs were aspirated from all the visible follicles(>2mm diameter) using a sterile 18G hypodermic needle attached to a 10ml disposable syringe containing 0.5-1 ml of aspiration medium. The fluid thus obtained having the COCs was allowed to settle for 15-20 minutes in sterile 90mm petridish at 37^0 C. After 15-20 minutes the supernatant was discarded and the pellet was resuspended in oocyte washing medium and screened for oocyte in 90mm petridish under stereomicroscope

Retrieval of oocyte by slicing techniques

Each ovary was held firmly with sterile artery forceps in a 90mm petridish containing oocyte collection medium (modified HEPES-buffered Tyrodes medium) and was sliced as per the standard technique described by Datta *et al.* (1993). The oocyte was screened under a stereo zoom microscope and transferred to 35mm petridish containing oocyte collection medium and then graded.

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) [33].

Maturation of oocytes

Total number of buffalo ovaries used in the present study was 673 and the numbers of oocytes recovered were 1084. Only grade A and B oocytes were used for IVM. 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) [19] 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 the rate of(10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} 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_2, at 39 °C and at humidity of about 95%.

Assessment of maturation of oocytes

Cumulus cell expansion

The maturation of oocyte was assessed based on the expansion of the cumulus mass. The degree of cumulus expansion was assessed by the morphology of the cumulus mass at the end point of in vitro maturation as described by

Kobayashi *et al.* (1994) [21] and Ravindranatha *et al.* (2002b). Briefly,

Degree 2 (full cumulus expansion): All the cumulus cells were homogeneously spread and clustered cells were no longer present;

Degree 1 (moderate cumulus cells expansion): 70 per cent cumulus cells were homogenously spread and clustered cells were still observed and

Degree 0 (slight or no expansion): cumulus cells were highly adherent to the zona pellucida. oocytes with cumulus expansion of degree 1 and 2 were considered matured. The maturation rate and expression rate was expressed as percentage of the total number oocytes cultured.

Polar body extrusion

The maturation of oocytes was further evaluated by identifying the first polar body in the perivitelline space when the oocytes were denuded after maturation.

Nuclear Maturation Status

The nuclear maturation status was evaluated by using Hoechst staining as described by Ebrahimi *et al.* (2010) [10]. At the end of maturation period a proportion of COCs were recovered from the culture and transferred to 1.5 ml tube containing 400 µl of trypsin-EDTA (0.1%) solution (Sigma, St. Louis, MO, USA) and vortexed for 1min to remove cumulus cells. The oocytes were then recovered under a stereozoom microscope and transferred to Hoechst solution (10 µg/ml) and incubated for 20 min at 39^0 C. The oocytes were washed 3 times in PBS and observed under epifluorescence microscope. The emitted fluorescent signal of Hoechst was observed at 350 nm to analyse the oocyte chromatin configuration as germinal vesicle (GV) and metaphase II (M II).

Results

Effect of melatonin on cumulus cell expansion:

The data on degree of cumulus expansion of buffalo oocytes with the supplementation of melatonin in IVM media are given in the Table 1, Plate 1 and Fig 1 as well. Fully expanded oocytes (Degree II) were 59.34, 66.60, 65.00, 58.85, and 49.63% in control, 10^{-4} M (Group I), 10^{-6} M (Group II), 10^{-8} M (Group III), 10^{-10} M (Group IV) and 10^{-12} M (Group IV) melatonin supplemented maturation media, respectively.

The degree of cumulus expansion of *in vitro* cultured oocytes in maturation media supplemented with different concentration of melatonin at degree 2 cumulus cell expansion were significantly higher (66.6±0.42 per cent) in Group II followed by Group III (65.60±0.31 per cent), Group I (59.34±0.37 per cent), Group IV (58.85±0.67 per cent), Group V (53.55±0.40 per cent) and (49.63±0.31 per cent) in control respectively.

The difference were highly significant ($p<0.01$) in Degree 2 when compared to other degrees of cumulus cell expansion recorded in matured oocytes.

In the present study apart from cumulus cell expansion in Group II out of 192 immature oocytes cultured 128 oocytes were matured followed by 124out of 189 immature oocytes in Group III. This showed that highest maturation of oocytes were recorded due to the effect of melatonin supplementation at 10^{-6}M and 10^{-8} M in Groups II and III respectively.

Table 1: Effect of supplementation of melatonin at different concentration on degree of cumulus cell expansion of in vitro matured buffalo oocytes

Groups	Treatment	Number of Oocytes cultured	Cumulus expansion, n% (mean ± S.E.)		
			Degree 0	Degree 1	Degree 2
Control	MM alone	163	42 (20.85 ± 0.21)	40 (24.54 ± 0.33)^a	81 (49.63 ± 0.31)^d
Group I	MM + 10^{-4} M, Melatonin	182	32 (11.53 ± 0.34)	42 (23.07 ± 0.31)^a	108 (59.34 ± 0.37)^b
Group II	MM + 10^{-6} M, Melatonin	192	19 (9.89 ± 0.31)	45 (23.48 ± 0.31)^a	128 (66.6 ± 0.42)^a
Group III	MM + 10-8 M, Melatonin	189	22 (11.64 ± 0.21)	43 (22.75 ± 0.21)^a	124 (65.60 ± 0.31)^a
Group IV	MM + 10^{-10} M, Melatonin)	175	32 (22.28 ± 0.22)	40 (22.85 ± 0.37)^a	103 (58.85 ± 0.67)^b
Group V	MM + 10^{-12} M, Melatonin	183	42 (22.95 ± 0.37)	43 (23.49 ± 0.43)^a	98 (53.55 ± 0.40)^c

Values with different superscript within column differ significantly ($P < 0.05$)

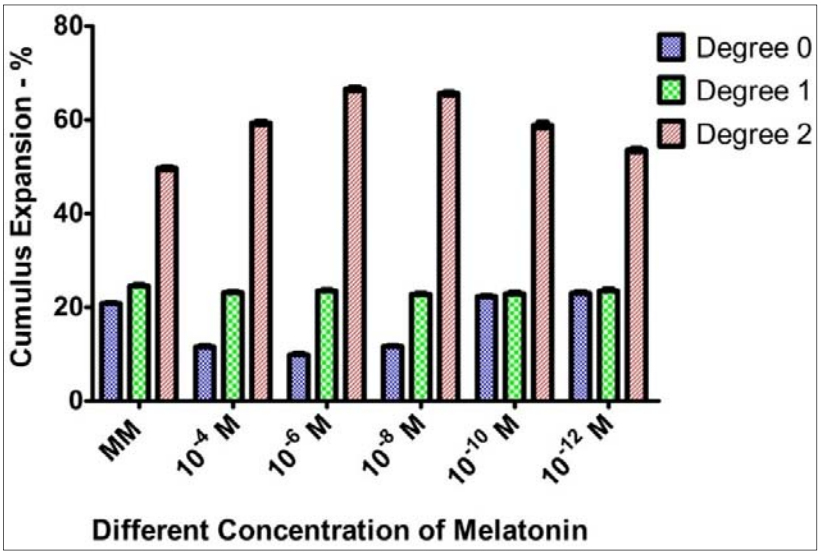
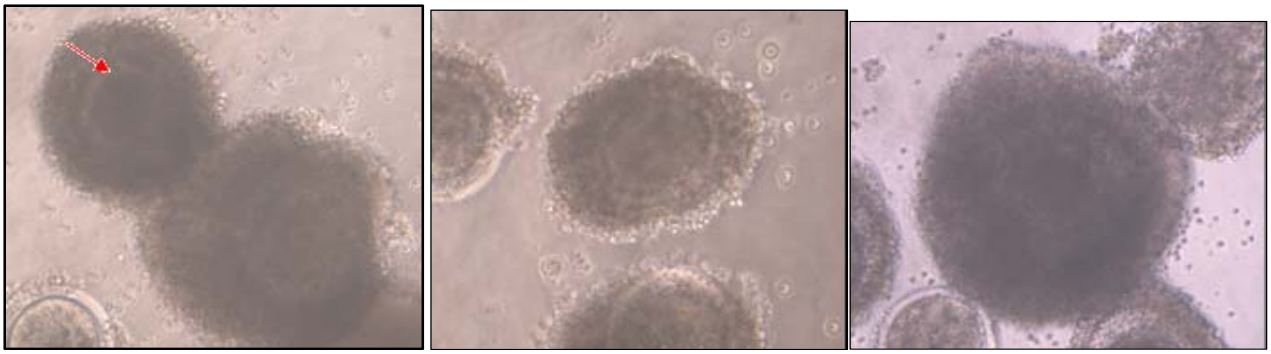


Fig 1: Effect of supplementation of melatonin at different concentration on degree of cumulus cell expansion of *in vitro* matured buffalo oocytes



2a: Degree 0: Slight or no cumulus expansion **2b:** Degree 1: Moderate cumulus expansion **2c:** Degree 2: Full cumulus expansion

Plate 1: Degree of cumulus expansion in *in vitro* matured buffalo oocytes (100x)

Effect of melatonin on extrusion of polar body of *in vitro* matured oocytes

The data on extrusion of polar body of buffalo oocytes with the supplementation of melatonin in IVM media are given in the Table 2, Plate2 and Fig 2 as well. Fully extrusion of polar body of oocytes were 81.31, 89.58, 88.36, 81.71, 75.41 and 73.00% in control, 10^{-4}M, 10^{-6}M, 10^{-8}M, 10^{-10}M and10^{-12}M melatonin supplemented maturation media, respectively.

The first polar body extrusion of *in vitro* cultured oocytes in maturation media supplemented with different concentration of melatonin were significantly higher (89.58± 0.79 per cent) in oocytes cultured in media supplemented with 10^{-6}M in Group II followed by 10^{-8}M (88.36±0.80 per cent) of melatonin supplemented Group III than in oocytes cultured in media supplemented with10^{-4}M(81.31±2.06per cent), 10^{-10} M (81.71±1.81per cent), 10^{-12} M (75.41±1.60 per cent) Group I, Group IV, Group V and (49.63±0.31per cent) in control respectively. The difference were significant ($p<0.05$) with Group III and II when compared to control.

Table 2: Effect of supplementation of melatonin at different concentration on polar body extrusion of in vitro matured buffalo

Groups	Treatment	No. of oocyte cultured	Polar body Extrusion
Control	MM alone	163	119 (73.00 ± 0.8)^{d}
Group I	MM +10^{-4} M Melatonin	182	148 (81.31 ± 2.06)^{b}
Group II	MM+10^{-6} M Melatonin	192	172 (89.58 ± 0.79)^{a}
Group III	MM +10^{-8}M Melatonin	189	167 (88.36 ± 0.80)^{a}
Group IV	MM+10^{-10} M Melatonin	175	143 (81.71 ± 1.81)^{b}
Group V	MM +10^{-12}M Melatonin	183	138 (75.41 ± 1.60)^{c}

Values with different superscript within column differ significantly ($P < 0.05$)

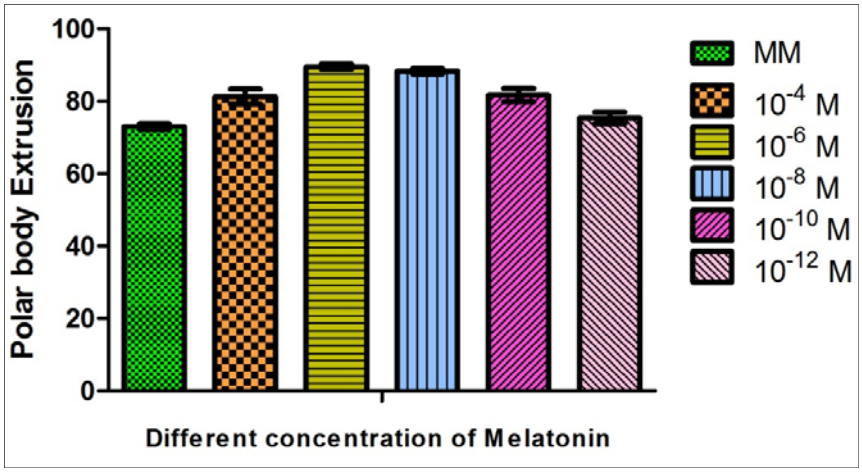


Fig 2: Effect of supplementation of melatonin at different concentration on polar body extrusion of *in vitro* matured buffalo oocytes

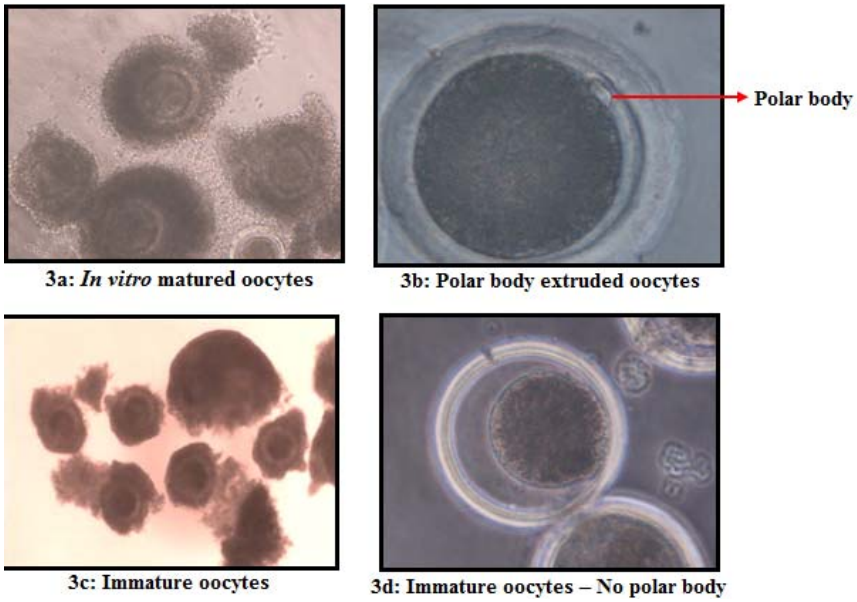


Plate 2: Polar body extruded (matured) and non extruded (immatured) oocytes

Discussion

Effect of Melatonin on Cumulus Cell Expansion of *in vitro* Matured Oocytes

In the present investigation the effect of melatonin supplementation was assessed by adding melatonin at dose levels of 10^{-4} M, 10^{-6} M, 10^{-8} M, 10^{-10} M and 10^{-12} M in the culture medium of oocytes and comparing the effects on the maturation of oocytes after 24 hrs of culture. In the present study 24 hrs time was followed in accordance with (Gasparrini, 2002) [11].

It was inferred by (Buccione *et al*., 1990, Armstrong, 1996) that assessment of cumulus cell expansion is an indicator of maturation of oocytes in IVM.

The most important harmful factors affecting oocytes maturation and embryo development were free radicals which have deteriorating effects on DNA repair, mitotic spindle assembly and maturation of oocytes (Agawal *et al*., 2006) [1].

Similar observation was made by Morado *et al.* (2009) [28], who claimed that free radicals cause damage to RNA, DNA, proteins, carbohydrates, lipids and further cause failure of mitochondria. According to him decreasing ROS molecules were necessary for *in vitro* oocyte maturation.

The cumulus cell expansion values are expressed as Degree 0, Degree 1 and Degree 2.

It was found that in all the groups Degree 2 of cumulus cell expansion were significantly higher when compared with Degree 0 and Degree 1. However it was observed that the cumulus cell expansion assessed were not of the same percentage in all the media groups. The n% (mean± S.E) observed was the highest in Group II (10^{-6} M) and III (10^{-8}M) and the values were 66.6 ± 0.42 and 5.60 ± 0.31 per cent respectively.

In the present study the number of matured oocytes obtained by supplementation of melatonin at the level of 10^{-6} M in Group II was 128 oocytes out of 192 immature oocytes followed by 124 out of 189 immature oocytes with 10^{-8}M in Group III.

This result revealed that the highest number of matured oocytes was recorded due to the effect of melatonin supplementation at 10^{-6} M and 10^{-8} M levels in Groups II and III respectively.

Since greater number of Degree 2 oocytes were recorded in all the groups supplemented with melatonin, it is inferred that melatonin supplementation augments the maturity level of oocytes *in vitro* culture and the same correspond with the following observation. Melatonin functions through multiple receptors, both membrane and nuclear level and also as a direct free radical scavenger, a process that requires no receptors was unequivocal (Hiroshi Tamura *et al*., 2009) [15].

From the values presented in the Table 4 it could also be seen that the highest number of Degree 2 oocytes are recorded in the Groups II and III with melatonin supplementation at 10^{-6} M and 10^{-8} M levels.

The result of this study showed that the optimum concentration of melatonin in IVM medium was 10^{-6} M and very low and very high doses have negative effects. Thus as reactive oxygen species in a controlled concentration was vital for oocyte maturation, very high and very low doses could be detrimental for oocytes during IVM. Therefore the concentration of antioxidants is crucial (Ishizuka *et al*., 2000) [17].

Further, this level plays a major role in preventing apoptosis by activating the receptors found in the cumulus cells. Similarly, cumulus cells are known to play a crucial role during oocyte maturation. Cumulus cells during maturation are essential for acquiring developmental competence by oocyte *in vitro* (Gordon, 2003) [12]. It was reported that 1µM melatonin reduce cumulus cell apoptosis by activating it receptors on cumulus cells (Na *et al*., 2005) [29].

Therefore it is also inferred that optimal level of melatonin supplementation for better level of buffalo oocytes maturation is 10^{-6} M as at this level of concentration maximum number of Degree 2 oocytes were recorded.

Effect of supplementation of melatonin on extrusion of polar body of *in vitro* Matured Oocytes

The number of matured oocytes with polar body extrusion were the highest in Groups II and III and the values were 172 (89.58 ± 0.79 per cent) and 167 (88.36 ± 0.80 per cent) and the lowest value was 119 (73.00 ± 0.80 per cent) in the control group. The differences observed between the control and other groups were significant ($p<0.05$).

Leibfried-Rutledge *et al.* (1987) [23] reported that nuclear maturation of oocytes commenced with germinal vesicle break down and concluded with completion of the first meiotic division and extrusion of the first polar body.

Since extrusion of polar body is considered as an indicator of the completion of oocytes maturation it is inferred from the values observed in this study that maximum number of oocytes attained maturity in the Group II and III.

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