

Effect of melatonin supplementation to the maturation media on *in vitro* cumulus cells expansion and nuclear maturation rates of buffalo oocytes

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ABSTRACT

The aim of this study is to determine the effect of the melatonin addition to the maturation medium (MM) on *in vitro* maturation of buffalo oocytes (IVM). Maturation is evaluated by the cumulus cells expansion and nuclear maturation. The cumulus oocyte complexes (COCs) were harvested by aspiration of antral follicles (2-8 mm diameter) of slaughter buffalo ovaries, COCs with homogeneous cytoplasm and at least three layers of cumulus cells were selected. The COCs were incubated for 24h in TCM-199 medium with melatonin (10, 30 or 50ng/ml) or without addition (control). After 24 h in culture media, the maturation rate of oocytes was determined by evaluating the expansion degree of the cumulus-oocyte complex (COCs) in each treatment, then classified into: fully expanded, partially expanded or not expanded, and by evaluation stages of oocyte nuclear maturation (GV, GVBD, MI, MII or degenerated). Results illustrate that supplementation maturation media with 10 and 30, 50 ng/ml melatonin resulted in increasing the cumulus cell expansion of buffaloes oocytes by 86.4, 82.3, 83.2 %, respectively, as compared with oocytes cultured with melatonin - free medium (73.3%), The respective differences were significant. Majority of this improvement in the expansion rates of oocytes was observed in the rate of fully expanded oocytes, On the contrary, the rate of immature (unexpanded) oocytes incubated in melatonin-free medium was significantly ($P<0.01$) higher (26.7%) than those incubated in maturation media with 10 or 30 or 50 ng/ml melatonin (13.8, 17.7, 16.9 %, respectively). Moreover, results indicated that addition of melatonin to the maturation medium significantly improved the percentage of oocytes of first polar body (matured to MII) as compared with those incubated without melatonin, the highest percentage of those oocytes was obtained with 10 ng/ml melatonin (84.5 %), however, the least percentage was in those oocytes cultured with melatonin - free medium (48.6%) with significant differences ($P<0.05$). On the other side, the percentage of oocytes of GVBD, MI or degenerated stages did not significantly affected with melatonin addition (10, 30 or 50 ng/ml) to culture media as compared with those cultured without melatonin medium. It could be concluded that addition of melatonin to the MM improved cumulus expansion and nuclear maturation rate of buffalo oocytes.

Key words: Buffalo, IVM, oocyte maturation, melatonin.

Introduction

As a matter of fact, buffaloes in Egypt represent the main source of milk and meat production. However, their reproductive potentially is still beyond optimum. Efforts have been initiated to improve the reproduction potential of these animals using modern biotechnologies such as., artificial insemination, *in vitro* maturation, fertilization and embryo transfer.

Oocyte maturation is one of the most important steps for successful *in vitro* production of bovine embryos (Dieleman et al., 2002). Oocyte *in vitro* maturation is intended to yield oocytes that normally could complete their first meiotic division to undergo normal fertilization, and would result in a zygote capable of full-term development after embryo transfer (Downs, 1993 and Royere 2006). Cumulus cells are known to play a crucial role during oocyte maturation. Gordon, (2003) reported that cumulus cells during maturation are essential for acquiring developmental competence by oocyte *in vitro*. Optimal expansion of the cumulus mass appears to be essential for cytoplasmic maturation (Testart et al. 1983, Poeggeler et al, 1993). In bovine, the induction of cumulus expansion prior to fertilization increased the incidence of oocyte penetration (Ball et al. 1983).

However, the *in vitro* maturation system is not as efficient as *in vivo* maturation (Hendriksen et al., 2000) because the *in vitro* handling and culture conditions expose oocytes and embryos to oxidative stress resulting from events such as light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates (Kitagawa et al., 2004., Agrawal et al., 2006). Moreover, It has been also speculated that buffalo oocytes / embryos, due to their high lipid content (Boni et al, 1992), are particularly sensitive to the increased oxidative stress that occurs under *in vitro* conditions (Gasparrini et al, 2003), causing damage effects on cellular structure such as mitochondria and microtubules and could disturb normal cell function (De Matos and Furnus 2000; Khatir et al. 2005). Therefore, it is important to protect oocytes from oxidative stress during *in vitro* maturation (IVM).

Melatonin (N-aceyl-5-methoxytryptamine), an indole derivative secreted rhythmically from the pineal gland, has major effects on the reproductive system in mammals (Sirotkin and Schaeffer, 1997; Reiter,1998), acts as a powerful free radical scavenger and likely to be a general promoter of anti-oxidative mechanisms and a potential antioxidant *in vitro* and *in vivo* (Reiter, 1998; Tan et al., 2007). Melatonin-treated oocytes had a significantly lower level of ROS

than controls. The presence of melatonin receptors in COCs and its promoting effects on oocyte nuclear and cytoplasmic events, indicate the potentially important roles of this hormone in regulating bovine oocyte maturation (El-Raey et al., 2011).

Melatonin is likely to become the drugs of choice for improving oocytes quality for woman who cannot get pregnant because of poor oocytes quality (Takazuki et al., 2003), as well as., exogenous melatonin has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM (Kang et al., 2008), and increased ratio of vitrified sheep blastocysts developed with melatonin application for 24 and 48 h *in vitro* was reported (Abecia et al., 2002). The presence of melatonin receptors in COCs and its promoting effects on oocyte nuclear and cytoplasmic events, indicate the potentially important roles of this hormone in regulating bovine oocyte maturation. Moreover, the presence of ASMT transcript in COCs suggests the possible involvement of these cells in melatonin biosynthesis (El-Raey et al., 2011).

It is worthy to call into attention, the degree of expansion of cumulus cell mass has been routinely considered in evaluating buffalo oocyte maturation *in vitro* (Palta and Chauhan, 1998 & Nandi et al., 2002), and for predicting subsequent *in vitro* fertilization success (Herrler et al., 1992). In addition, the maximum cumulus expansion and extrusion of first polar body is expected to occur during 22-24h of culture (Nandi et al., 2002).

Therefore, the objective of this study was to evaluate the effects of melatonin supplementation to the maturation medium on the cumulus cells expansion and nuclear maturation of buffalo oocytes *in vitro*.

Materials and Methods

Ovaries collection:

Buffalo ovaries were collected from Shebin El Kom slaughterhouse and were placed immediately after slaughtering into thermos in phosphate buffer saline (PBS) containing 50 µg/ml gentamycin, and transported to the laboratory in Sakha, Kafr El-Sheikh governorate within 2 h at 30-34°C. Thereafter; ovaries were washed twice in phosphate buffer saline to remove any adhering clotted blood. The previous history of the reproductive tract of slaughtered animals was unknown.

Oocyte recovery:

The cumulus oocyte complexes (COCs) with dark homogeneous cytoplasm and at least three layers of cumulus cells as described by De Loos et al. (1989) were harvested by aspiration of antral Follicles of 2-8 mm diameter using an 18-gauge needle attached to 10 ml syringe. The COCs were examined under a stereomicroscope.

In vitro maturation (IVM) and experimental treatments:

• Preparation of maturation medium:

On the day of oocyte investigation, maturation medium was prepared. The maturation media consisted of TCM 199 supplemented with 10% estrous buffalo serum, 10 µg/ml FSH, and 5 µg/ml LH, 1 µg /ml estradiol 17 β , 3 mol L⁻¹ sodium pyruvate and, 50µg/ml gentamycin sulfate . The maturation medium was supplemented with either 0 or 10 or 30 or 50 ng/ml Melatonin. The medium was adjusted to pH of 7.2-7.4 and osmolarity level of 280-300 mOsmol/kg and filtered by 0.22 µm-millipore filter. (All chemicals and supplementations were purchased from sigma chemical Co).

About 200 µl from each prepared maturation medium was placed into sterile Petri dish and covered by sterile mineral oil. Before oocytes were placed in culture dish, these dishes were incubated in CO₂ incubator (5% CO₂) at 39°C and high humidity for one hour at least for equilibration.

• Oocytes maturation:

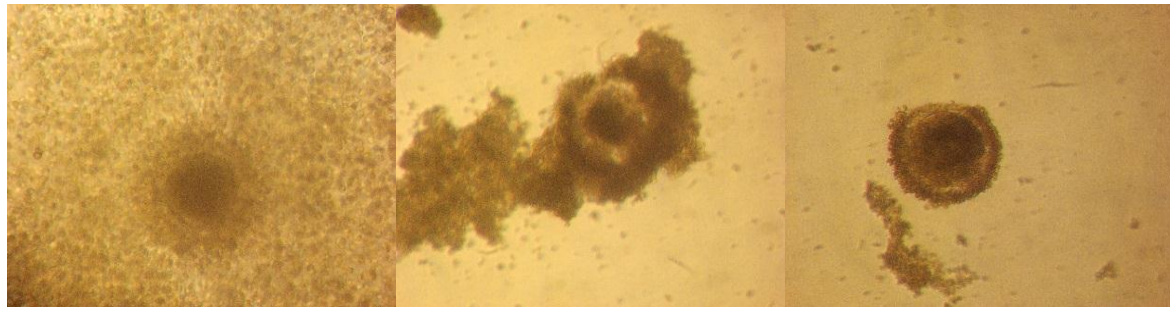
The cumulus oocyte complexes were rinsed three times in one of the intended maturation media before culturing in that medium, and incubated in groups of 10-15 oocytes in a 100 µl droplet of each culture maturation medium. The droplets were covered with mineral oil in 35 mm dish and incubated for 24h at 38.5°C in an atmosphere of 5% CO₂ in humidified air.

Evaluation of oocyte maturation:

A. Oocyte expansion:

After 24 h in culture, the expansion degree of the cumulus-oocyte complex (COCs) in each treatment of maturation medium was determined to evaluate the efficiency of each medium which was classified as follow:

- Fully expanded (all cumulus cells were loosened)
- Partially expanded (the outer layer of cells was loosened) or
- Not expanded. (As shown in Fig. 1)



(A) fully expanded, (B) partially expanded, (C) not expanded.

Figure 1. Grades of cumulus cell expansion in COCs after 24h incubation in maturation media.

B. Evaluation of oocyte nuclear maturation

At the end of maturation period, COCs were removed from the maturation medium and the cumulus cells were removed from the oocytes,.

Fixation of the oocytes was carried out by placing the slides in a fresh mixture of acetic acid: ethanol (1:3) overnight (Sun et al., 2001). Oocytes were then stained with 1% (w/v) aceto-orcein. for a few minutes and washed by aceto-glycerol (3:1). Both low power and oil immersion were used for detailed examination. Oocytes were categorized as follows:

Criteria of nuclear maturation:

After maturation of the oocytes, they were categorized as follows:

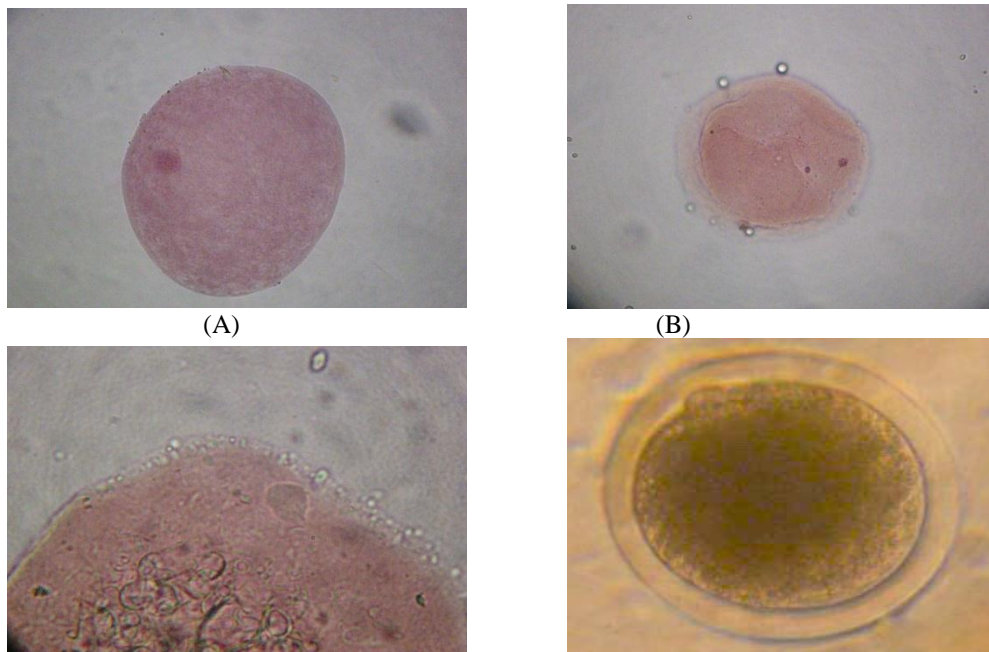
a. Germinal vesicle (GV): Inter phase chromosomes enclosed within a nuclear membrane.

b. Germinal vesicle breakdown (GVBD): An absence of a visible nuclear membrane and chromatin condensation characterized by a cluster of DNA material without individual chromosomes.

c. Metaphase I (MI): Chromosomes were condensed in pairs and without detected polar body.

d. Metaphase II (MII): One the larger group of chromosome formed an equatorial plate and the remaining chromosome are highly condensed or had extruded a polar body (mature oocyte).

e. Degenerated: Oocytes were vacuolated or had scattered or highly condensed chromatin. (As shown in Fig. 2)



(A) Germinal vesicle stage (GV) (B) Germinal vesicle breakdown (GVBD) (C) Metaphase I (D) Metaphase II.

Fig.2: criteria of nuclear maturation of matured oocytes

Statistical analysis:

The obtained data were subjected to one way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine significant difference among means at the $p = 0.05$ level (Duncan, 1955).

Results and Discussion**The effect of melatonin supplementation on maturation rate of buffalo oocyte:**• **Oocyte expansion:**

Results in Table 1 demonstrate that supplementation maturation media with 10 and 30, 50

ng/ml melatonin resulted in increasing the cumulus cell expansion of buffaloes oocytes by 86.4, 82.3, 83.2 %, respectively, as compared with oocytes cultured with melatonin - free medium (73.3%). The respective differences were significant. Majority of this improvement in the expansion rates of oocytes was observed in the rate of fully expanded oocytes, which ranged from 12.2 to 18.9% as compared with those cultured in melatonin-free medium (38.8%). On the contrary, the rate of immature (unexpanded) oocytes incubated in melatonin-free medium was significantly ($P < 0.01$) higher (26.7%) than that incubated in maturation media with 10 or 30 or 50 ng/ml melatonin (13.8, 17.7, 16.9 %, respectively).

Table 1. Effect of melatonin supplementation to maturation medium on expansion rate of buffalo oocytes.

melatonin conc. (μm)	Total tested oocytes	Cumulus expansion							
		Full expanded		Partially expanded		Maturation rate (Full+Partially expanded)		Non expanded	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Control	206	80	38.8 \pm 1.1 ^c	71	34.5 \pm 0.9 ^a	151	73.3 \pm 0.9 ^a	55	26.7 \pm 1. ^a
10 ng/ml	189	109	57.7 \pm 0.9 ^a	54	28.6 \pm 1. ^{0c}	163	86.4 \pm 1.1 ^c	26	13.8 \pm 1.1 ^c
30 ng/ml	198	101	51.0 \pm 0.9 ^b	62	31.3 \pm 1.1 ^b	163	82.3 \pm 1.2 ^b	35	17.7 \pm 0.7 ^b
50 ng/ml	178	98	55.06 \pm 1.3 ^a	50	28.1 \pm 0.9 ^c	148	83.2 \pm 0.9 ^b	30	16.9 \pm 1.3 ^b

a, b and c: Means denoted within the same column with different superscripts are significantly different at $P < 0.001$.

The obtained maturation rate in the present study characterized by expansion of COCs is higher than that reported by Totey et al. (1991, 1992 and 1993) (40, 63.6 and 80.0 %, respectively), Jainudeen et al. (1993) 47.0%, Chauhan et al. (1998) 82.0 % and Nandi et al. (2003) 83.0 % in buffaloes. It has been reported that type of medium and supplements which is used for IVM, may influence rate of cumulus cell expansion (Gordon, 2003).

• **Nuclear maturation of oocytes**

Generally, the tabulated data Table 2 indicated that addition of melatonin to the maturation medium significantly improved the percentage of oocytes of first polar body (matured to MII) as compared with those incubated without melatonin, the highest percentage of in those oocytes was obtained with 10 ng/ml melatonin (58.7 %), however, the least percentage was in those oocytes cultured with melatonin - free medium (48.6%) with significant differences ($P < 0.05$).

Table 2. Effect of melatonin supplementation to maturation medium on nuclear maturation of buffalo oocytes in vitro.

Item	N	Maturation stage of buffalo oocytes									
		GV		GVBD		M I		M II		Degen.	
		N	%	N	%	N	%	N	%	N	%
Control	202	20	9.3 \pm 1.8	26	12.7 \pm 1.1	38	18.7 \pm 1.5	96	48.6 \pm 2.8 ^b	22	10.8 \pm 1.3
10 ng/ml	189	16	8.4 \pm 1.5	16	8.5 \pm 1.4	29	15.0 \pm 1.7	110	58.7 \pm 3.1 ^a	18	9.3 \pm 1.6
30 ng/ml	197	22	10.9 \pm 1.3	22	11.1 \pm 1.1	37	18.5 \pm 1.8	96	49.9 \pm 2.8 ^b	20	9.6 \pm 1.8
50 ng/ml	184	20	11.0 \pm 2.0	19	10.3 \pm 1.4	33	17.9 \pm 1.7	92	49.8 \pm 3.0 ^b	20	11.0 \pm 1.7 ^{NS}

a, b and c: Means denoted within the same column with different superscripts are significantly different at $P < 0.05$.

It appears that, the percentage of oocytes of GVBD, MI or degenerated stages did not significantly affected with melatonin addition (10, 30 or 50 ng/ml) to culture media as compared with those cultured without melatonin medium (Table 2).

In conclusion, our results proved that supplementation maturation media with 10 and 30, 50 ng/ml melatonin resulted in increasing the rates of cumulus cell expansion and nuclear maturation of buffalo's oocytes as compared with those cultured without melatonin addition, this is consistent with the results of Manjunatha et al. (2007) who reported that, addition of melatonin by 10 μ M to 50 μ M, increase nuclear maturation and transformable embryo of buffalo oocyte *in vitro*. Leona et al., (2004) stated that melatonin can decrease H₂O₂ level in mitochondria, elevates glutathione homeostasis and improves mitochondria performance and activates repairing mechanism of mitochondria genome.

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