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Effects of dibutyryl cyclic adenosine monophosphate (dbcAMP) on growth and survival of buffalo oocytes

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ABSTRACT

The function of cyclic adenosine monophosphate (cAMP) is to maintain the meiotic arrest in mammalian oocytes. This study was undertaken to determine the effects of dibutyryl cyclic Adenosine 3',5' -monophosphate (dbcAMP) on *in vitro* growth and survival of buffalo oocytes. Cumulus-oocyte-complexes (COCs) collected from early antral follicles were cultured for 14 days in growth culture medium dulbecco's minimum essential medium supplemented with 0, 0.5, 2.5-, and 10-mM level of dbcAMP on collagen gel in 96-well culture plate at 38.5°C under an atmosphere of 5% CO_2 in air. After 14 days 14.7, 5.9 and 5.9% oocytes become denuded; 67.6, 85.3 and 82.4% remained enclosed and 5.9, 5.9 and 5.9% were degenerated in 0.5, 2.5 and 10.0 mM dbcAMP supplemented culture, respectively. All oocytes were denuded in dbcAMP free group. After 14 days of culture 29.4, 52.9 and 41.2% oocytes increased over 116 µm; 44.1, 29.4 and 41.2% oocytes increased diameters between 100 to 115 µm and 14.7, 5.9 and 5.6% oocytes remain below 100 µm for 0.5, 2.5 and 10 mM dbcAMP supplemented culture respectively. After maturation 83.3, 50, 30.3 and 37.5% oocytes remain Germinal Vesicle (GV) stage and 16.7, 50, 67.6 and 68.8% oocytes went to meiosis I (MI) stage for 0, 0.5, 2.5 and 10 mM dbcAMP supplemented culture respectively, but none of them developed to the Meiosis II (MII) stage. Moreover *In vivo* grown oocytes were developed 37.5% to MII stage. The results showed that dbcAMP were maintained the integrity of oocytes and surrounding cumulus cells. In conclusion, during buffalo oocyte IVM the dbcAMP prevents denudation and degeneration of oocytes and increases survivability in culture by maintaining the meiotic arrest of oocytes and also increases the diameter of oocytes.

Introduction

The buffalo (*Bubalus bubalis*) plays a vital role in livestock agriculture across Asia, serving as a source of draft power, milk, meat, and hides. The global buffalo population is approximately 199.8 million, with over 97 per cent residing in the Asian regions (FAO, 2013). Bangladesh has an estimated buffalo population of around 1.524 million (DLS, 2023). Most of them are unknown types of river buffaloes. Buffaloes are known to have low reproductive efficiency, commonly exhibiting issues such as silent estrus, seasonal anestrus, delayed puberty and first calving, prolonged postpartum intervals, and extended calving intervals (Nandi *et al.,* 1997). Because of poor response to multiple ovulation and embryo transfer (MOET) treatments (Zicarelli *et al*., 1997), interest has been increasing in the *in vitro* embryo production (IVEP) technology in buffaloes. The problem of IVEP technology in buffalo is the lower number of immature oocytes that can be harvested from each donor cow. On average, follicular aspiration from abattoir-derived buffalo ovaries results 2-3 good-quality oocytes per ovary, compared to approximately 10 in cattle (Gordon, 1994). Similarly, small numbers of oocytes are found when ovum pick up (OPU) is performed in buffaloes compared to cattle (4.5 vs

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approximately 10 respectively; Galli *et al.,* 2001). This limitation stems from the species' physiological characteristics, including a low number of antral follicles on the buffalo ovary and a high incidence of follicular atresia. (Palta and Chauhan, 1998). To overcome the shortage of large follicles, attempt should be taken to grow up oocytes in smaller follicles to the full size *in vitro*. Oocyte maturation and oocyte quality are fundamental to fertility in all mammalian species. Gupta *et al*. (2008) produced buffalo embryos by *in vitro* fertilization of oocytes derived from *in vitro* grown preantral follicles. Various growth factors and cytokines are known to play key roles in regulating germ cell or oocyte survival and follicular development (Reynaud and Driancourt, 2000; Eppig, 2001).

The functional unit within the ovary is the follicle, which is comprised of one or more layers of granulose cells surrounding the oocyte (Zeleznik, 2004). Ovarian follicles are formed during embryonic development (Gougeon, 1996; Eppig *et al*., 2004). During follicular growth, the somatic cells divide to form several layers around the oocyte, which increases in size and a fluid-filled antrum starts to develop. Oocyte maturation is the process through which the oocyte completes the first meiotic division, undergoes cytoplasmic changes, and progresses to the metaphase II stage. Although fully grown oocytes are capable of maturing, they remain arrested in prophase I until triggered by the luteinizing hormone (LH). It is well established that meiotic arrest is regulated by cAMP levels within the mammalian oocyte (Conti *et al*., 2002; Eppig *et al*., 2004). It has long been recognized that one of the most important classes of molecules regulating mammalian oocyte maturation are the cyclic nucleotides, namely, cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP). Among these, cyclic AMP (cAMP) has been a focal point of intensive oocyte research over the past four decades (Gilchrist *et al.,* 2016). cAMP plays a crucial role in intracellular signal transduction, acting as a secondary messenger in response to gonadotrophin stimulation. Recent studies (Leal *et al.,* 2018; Gupta and Nandi, 2010; Gupta *et al.,* 2011; Sharma *et al.,* 2009; Nandi *et al.,* 2009 and Islam *et al.,* 2019) suggest that stem cell factor (SCF) plays important roles in the growth, viability and nuclear maturation of buffalo oocytes *in vitro*. Estradiol has been shown to improve oocyte nuclear maturation in both buffaloes and goats (Maksura *et al.,* 2021). Hirao *et al*. (2004). Moreover, cyclic adenosine monophosphate (cAMP) modulator supplementations, growth factors and beta mercaptoethanol and androgens are important for the growth and survival of buffalo preantral follicles (PFs). A number of compounds can arrest oocytes at the germinal vesicle (GV) stage (Schultz *et al*., 1983; Kubelka *et al*., 2000). For example, dbcAMP, an analog of cyclic AMP (cAMP), is an important regulator of meiosis in oocytes. The earliest report on the role of cAMP utilized the membrane-permeable analog dibutyryl cAMP (dbcAMP), showing that oocytes can be maintained in meiotic arrest *in vitro* after being removed from their follicular environment (Cho *et al.,* 1974). It is hypothesized that treating prepubertal oocytes with dbcAMP during *in vitro* maturation (IVM) increases the cAMP levels (Bagg *et al*., 2006), which is associated with a slower meiotic progression and enhanced cytoplasmic maturation and developmental competence. It has been reported that dbcAMP maintain the meiotic arrest of porcine oocytes and promoted their growth *in vitro* (Cyo-colca *et al*., 2011).

A few reports are available on *in vitro* development of oocytes in indigenous buffaloes in Bangladesh. The purpose of this study was to examine the effects of dbcAMP treatment on growth and nuclear maturation of buffalo oocytes after *in vitro* growth culture.

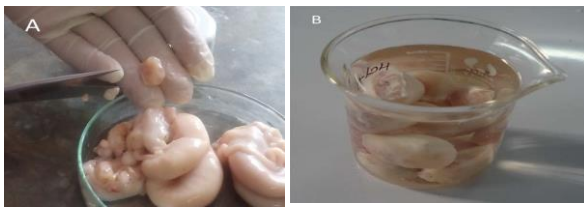
Materials and Methods

Chemicals and reagents

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned. The disposable media, chemicals and reagents were prepared before starting of the experiment. The experiment was conducted at the Animal Science Laboratory under the Department of Animal Science, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Processing of ovaries and collection of COCs

Ovaries were collected from healthy indigenous river buffaloes from Kaptan bazaar slaughter house, Dhaka. Processing of ovaries and the collection of COCs are presented in Figure 1 (A to F).



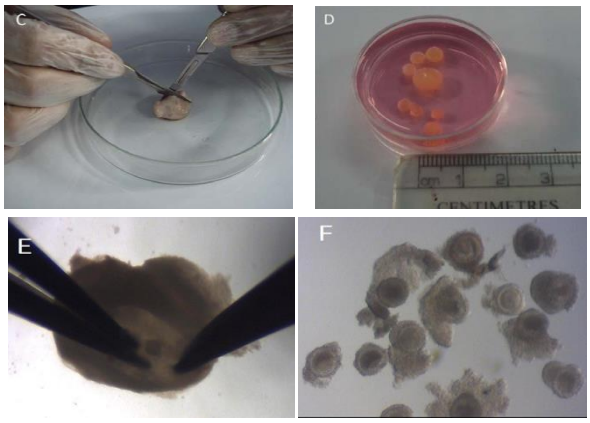


Figure 1: Collection of cumulus-oocyte complexes (COCs) from buffalo ovaries. The representative photographs were: (A) Separation of ovaries from oviducts; (B) Ovaries in PBS solution; (C) Dissection of follicles from ovaries; (D) Follicles in HEPES medium; (E) Opening of follicles to collect oocytes granulosa cell complexes; (F) Collected cumulus oocyte granulosa cell

The ovaries were stored in a thermo flask and transported to the laboratory within 3 to 4 hours. Corpus Luteum (CL) free ovaries were used because they contained a large proportion of early antral follicles and were not influenced by the hormonal effects of the cyclic changes of the estrus cycle. Following three washes in Dulbecco's phosphate buffered saline (DPBS), intact healthy early antral follicles (about 0.5 mm), which contained growing oocytes, and large antral follicles (4.0 to 6.0 mm in diameter) which contained fully grown oocytes, were dissected from the ovaries.

For collection of COCs with growing oocytes, ovarian cortical slices (2 mm thick) were made using a surgical blade (Keisei Medical Industrial Co., Ltd., Tokyo, Japan; No. 21) and a pair of forceps. Using a dissecting microscope, early antral follicles were isolated from the ovarian cortex, and the surrounding tissues were carefully sliced in PBS. Early antral follicles were opened with forceps and a needle in a culture dish (No. 1008, Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing 2 mL of 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) 10 mM sodium bicarbonate, and 0.1 mg/mL Gentamycin. To collect COCs containing fully grown oocytes, large antral follicles (4.0 to 6.0 mm in diameter) were dissected from the ovaries in PBS using two surgical blades (Keisei Medical Industrial Co., Ltd, Tokyo, Japan; No.11), and opened in medium in another dish. Collected COCs with growing oocytes from early antral follicles were used for growth culture and COCs with fully grown oocytes from large antral follicles were separately subjected to *in vitro* maturation.

Culture of oocyte cumulus complex from early antral follicles

The culture medium used for oocyte growth was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT, USA), 50 µg/mL L-ascorbic acid, 4% (w/v) PVP, 1 mM sodium pyruvate, 0.1 mg/mL Gentamycin, 4 mM hypoxanthine, 55 µg/mL cysteine and 0.05 µM dexamethasone (Sigma Aldrich, USA), 10 µg/mL estradiol-17β, 10 µg/mL androstenedione (Sigma) according to the method used by Taketsuru *et al.* (2012).

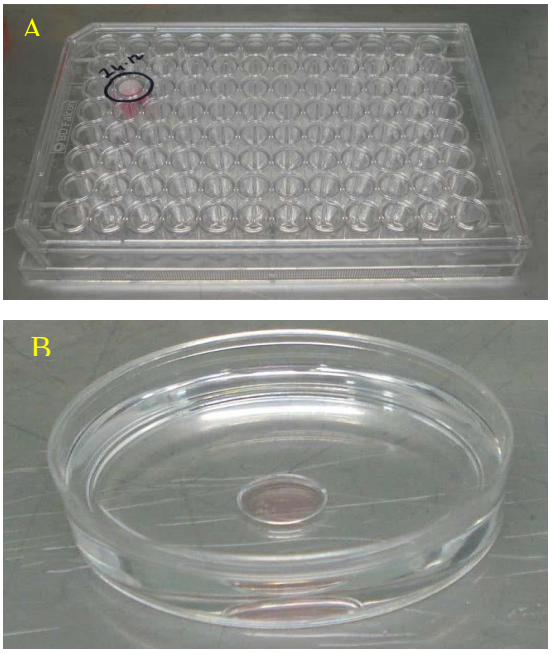


Figure 2: Buffalo cumulus-oocyte complexes (COCs) were cultured in 96 well dish (A) for growth and in droplet of culture medium (B) for maturation

To elucidate the effects of dbcAMP, the basic medium was supplemented with 0, 0.5, 2.5 and 10 mM dbcAMP (Sigma, USA). Each complex was transferred into culture dishes (Figure 2A; 96-well culture plate; Biocoat Collagen I Cellware, Falcon 354407, Becton Dickinson Falcon, Franklin Lakes, NJ, USA), which were coated with a collagen mixture consisting of 0.3% (w/v) collagen solution (Cellmatrix Type I; Nitta Gelatin, Osaka, Japan) prepared according to the manufacturer's instructions. The day of complex isolation was designated as Day 0. The complexes were cultured in 0.2 mL of medium at 38.5°C in an atmosphere of 5% CO_2 in air.

On Day 7 oocyte-granulosa cell complexes showing degenerative signs, such as cytoplasmic degeneration of oocytes and/or

complete detachment of granulosa cells from oocytes resulting in degradation of the complex structures were classified as degenerative complexes and excluded from further examination. The diameter of each oocyte, excluding the zona pellucida, was measured to the nearest 0.5 µm with an ocular micrometer (LABOMED, CZM6, IVu 5100) on 0, 7 and 14 days. Half of the medium was changed on 3rd, 6th, 9th and 12th days. After the size measurement on 14th day, the oocytes and surrounding granulosa cells were picked up from a 96-well culture plate and transferred to micro drops prepared for the induction of oocyte maturation as described below. Oocytes with intact morphology, characterized by the presence of firmly attached granulosa cells and no visible signs of degeneration, were considered viable and selected for *in vitro* maturation.

***In vitro* maturation (IVM) of oocytes**

The maturational competence of oocytes cultured *in vitro* was evaluated. After the 14-day culture period, oocytes were transferred into a 50 µL micro drop of the maturation medium (Figure 2B), which consisted of TCM-199 supplemented with 0.1 mg/mL sodium pyruvate, 0.08 mg/mL Gentamycin sulfate, 5% (v/v) FBS and 100 ng/mL follicle stimulating hormone (FSH; NIDDK, Washington, DC, USA) according to method used by Taketsuru *et al.* (2012). The oocytes were cultured at 38.5°C for 22 to 24 hours under an atmosphere of 5% CO_2 in air. At the end of the culture period, oocytes were mechanically denuded of granulosa cells using a small-bore pipette in the presence of hyaluronidase (Sigma). The oocytes were then mounted on a slide and fixed with acetic alcohol. The following day, the chromatin was stained with acetic orcein and examined under a differential interference contrast (DIC) microscope (Olympus).

Statistical analysis

All data were subjected to one-way ANOVA, and the significance of difference among means was determined using Duncan's multiple range test (DMRT). All statistical analyses were performed using SAS/STAT software, version 9.1.3 for Windows (Service Pack 4; SAS Institute Inc., Cary, NC, USA, 2004). Differences were considered statistically significant at $p<0.05$.

Results and discussion

Effect of dbcAMP on growth of buffalo oocytes *in vitro*

Buffalo ovaries contain a limited number of large follicles (4 to 6 mm) that contain full-grown oocytes (approximately 120 µm in diameter) but a substantial number of growing follicles with oocytes around 100 µm are present in each ovary (Gasparrini *et al.,* 2002). We also found a small number of full-grown oocytes and a large number of small oocytes in early antral follicles in each buffalo ovaries (Figure 3).

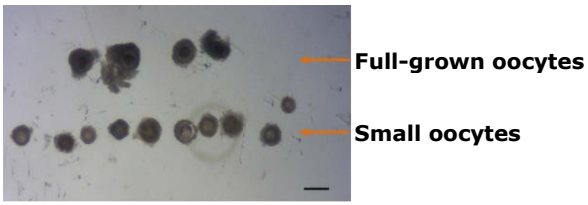


Figure 3: Buffalo cumulus-oocyte complexes (COCs) after *in vitro* maturation. Scale bar represents 100 µm.

These phenomena provided a basis of *in vitro* growth of small oocytes for *in vitro* embryo production. In the present study, the diameters of oocytes ranged from 90 to 100 µm before culture (Figure 4 and Figure 6). After growth culture on collagen gel, oocytes started to grow but their granulosa cells detached from the oocytes. The oocytes became denuded and finally degeneration was occurred in a week (Figure 4; Figure 5 and Table 1).

Table 1. Effect of dbcAMP on morphology of cumulus oocyte complex

Observation at	Morphology of cumulus oocyte complexes	Percentage (number) of oocytes at different concentration of dbcAMP			
		0 mM	0.5 mM	2.5 mM	10 mM
0 day	Denuded	0(0)	0(0)	0(0)	0(0)
	Enclosed	100 (34)	100 (34)	100(34)	100(34)
	Degenerated	0(0)	0(0)	0(0)	0(0)
7 days	Denuded	99.1(32)	5.9(2)	0(0)	2.9(1)
	Enclosed	5.9(2)	88.2(30)	97.1(33)	99.1(32)
	Degenerated	47.1(16)	5.9(2)	2.9(1)	5.9(2)
14 days	Denuded	(0)	14.7(5)	5.9 (2)	5.9(2)
	Enclosed	(0)	67.6 (23)	85.3(29)	82.4 (28)
	Degenerated	(0)	5.9(2)	5.9(2)	5.9(2)

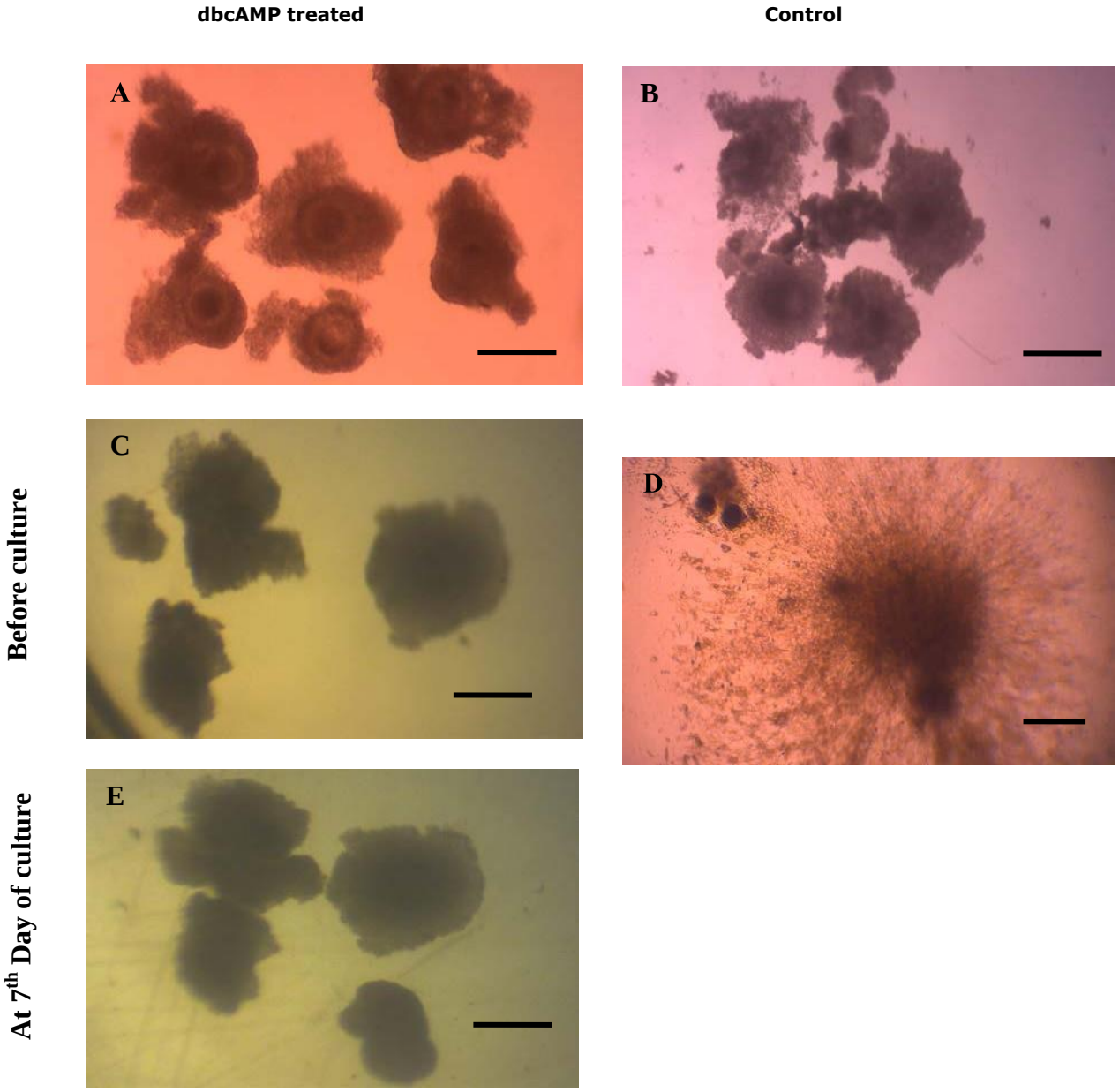


Figure 4: Morphology of COCs in culture media with dbcAMP (A, C and E) and without dbcAMP (B and D), respectively. Scale bars represent 100 µm.

Oocytes spontaneously resume meiosis once they are removed from their follicular environment (Carrol *et al.,* 1991). Thereby cumulus expansion is occurred and oocytes cannot be cultured for a long. So, during growth, oocytes need to remain in meiotic arrest. They can be suppressed with dibutyryl cyclic adenosine 3′ 5′ -monophosphate (dbcAMP). Therefore, dbcAMP was added into culture media to prevent the cumulus expansion of oocytes. The result of the present study showed that oocytes remained enclosed tightly by the cumulus cells in the dbcAMP supplemented media (Figure 4C; Figure 4E and Figure 5) and thus suggested that dbcAMP prevented the oocytes from denudation and degeneration of oocytes *in vitro*. In addition, supplementation of medium with dbcAMP significantly reduced degenerated oocytes compared to the control (Figure 5). A lower concentration (0.5 mM) of dbcAMP even promoted the growth of oocytes (Table 2 and Figure 6).

Table 2. Effect of dbcAMP on oocyte growth *in vitro*

Observation at	Diameter (µm) of oocytes	Number of oocytes at dfifferent concentration of dbcAMP			
		0 mM	0.5 mM	2.5 mM	10 mM

0 day	<100	34	34	34	34
	100-115	0	0	0	0
	>116	0	0	0	0
7 days	<100	30	25	20	21
	100-115	4	8	8	8
	>116	0	1	6	5
14 days	<100	-	5	2	2
	100-115	-	15	10	14
	>116	-	10	18	14

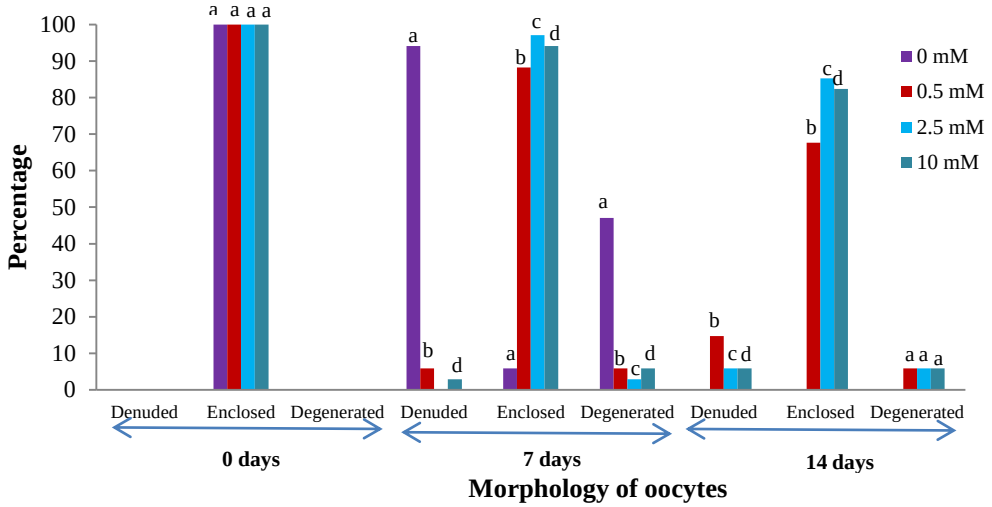


Figure 5: Effect of dbcAMP on morphology in percentage of cumulus-oocyte complexes (COCs). [a-d]Bar with uncommon superscripts differ significantly (p<0.05).

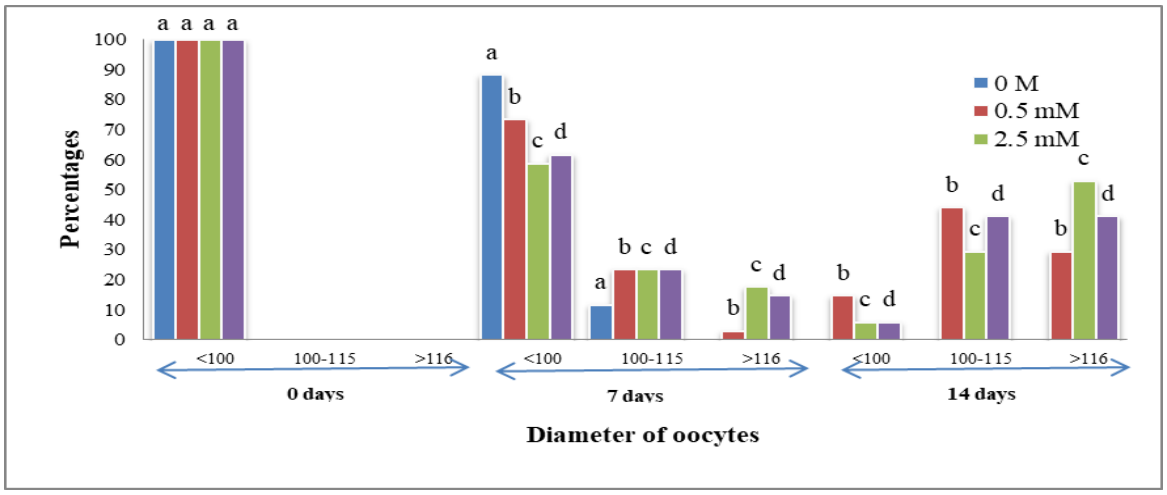


Fig 6: Effect of dbcAMP on oocyte growth in vitro. [a-d]Bar with uncommon superscripts differ significantly (p<0.05).

However, Cyo-colca *et al.* (2011) reported that dbcAMP maintain the meiotic arrest of porcine oocytes and promoted their growth *in vitro*. It is known that during growth, oocytes accumulate water, ions, carbohydrates, and lipids that are fundamental for nuclear and cytoplasmic maturation (Pederson and Peters, 1968).

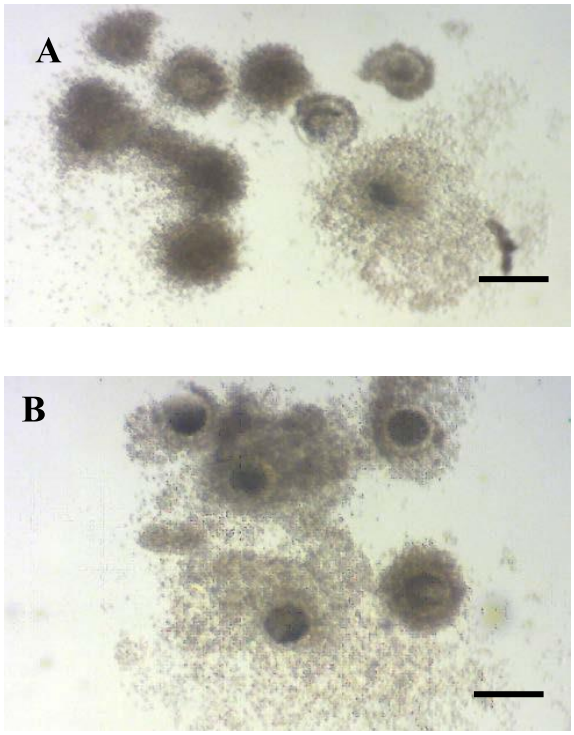


Figure 7: Buffalo cumulus-oocyte complexes (COCs) after maturation culture. From top to down- a number of *in vitro* grown oocyte showed cumulus expansion (A) while the expansion was observed in all *in vivo* control oocytes (B), respectively. The *in vitro* maturation medium was supplemented with 0, 0.5, 2.5 and 10 mM of dbcAMP. Scale bars represent 200 µm.

Cumulus cells secrete paracrine factors (Erickson and Shimasaki, 2001) and supply several known and unknown factors to the oocyte for growth and development (Brower and Schultz, 1982). The effects of gonadotropins are primarily mediated by changes in cAMP levels, which regulate the expression of specific genes during oogenesis (Richards and Hedin, 1988). In addition to maintaining meiotic arrest of growing oocytes, dbcAMP improved oocyte growth (Carroll *et al*., 1991) and increased steroidogenesis (Hsueh *et al.,* 1984), as well as secretion of some growth factors by granulosa cells (Hsu and Hammond, 1987).

The dbcAMP induced several fold proliferations of the granulosa cells is a likely explanation for improved conditions of oocyte growth.

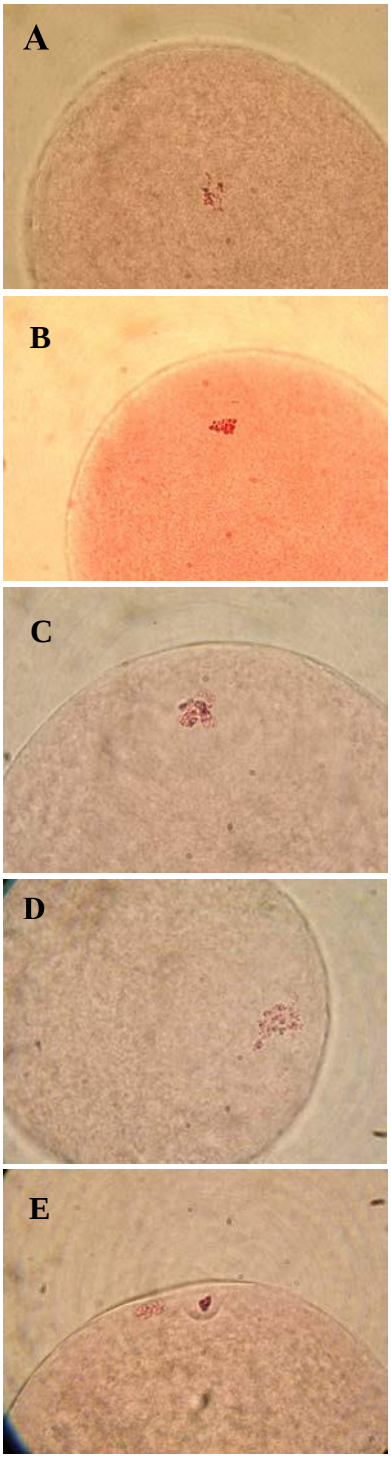


Figure 8. Nuclear morphology of oocytes after maturation culture. The *in vitro* maturation medium was supplemented with 0, 0.5, 2.5 and 10 mM of dbcAMP.

Oocyte growth is dependent upon gap-junction-mediated metabolic coupling with granulosa cells (Brower and Schultz, 1982; Herlands and Schultz, 1984), oocytes denuded of their granulosa cells and cultured separately, or when co-cultured with these cells (Eppig, 1979; Bachvarova *et al.,* 1980) or with other cell types (Canipari *et al.,* 1984; Herlands and Schultz,

1984; Buccione *et al.,* 1987), oocytes exhibit minimal, if any, growth.

Since oocyte growth is a phase of intense metabolic activity, an increase in the number of surrounding granulosa cells may facilitate the delivery of essential nutrients. *In vivo*, early follicular development involves oocyte growth with a concomitant increase in numbers of granulosa cells (Pederson and Peters, 1968). The observation that granulosa cell proliferation is associated with the improved rates of oocyte growth *in vitro* suggests that oocyte growth and follicular development are interrelated. Including dbcAMP in the culture medium may better replicate the physiological conditions found *in vivo*. cAMP is the putative second messenger for follicle stimulating hormone (FSH) and the concentrations of FSH are known to be high in the serum of juvenile mice (Michael *et al.,* 1980). FSH also stimulates granulosa cell mitosis in small follicles *in vivo* (Ryle, 1972; Pederson, 1970) and *in vitro* (Roy and Greenwald, 1986; 1989); thus, the endogenous hormone and dbcAMP appear to have similar effects.

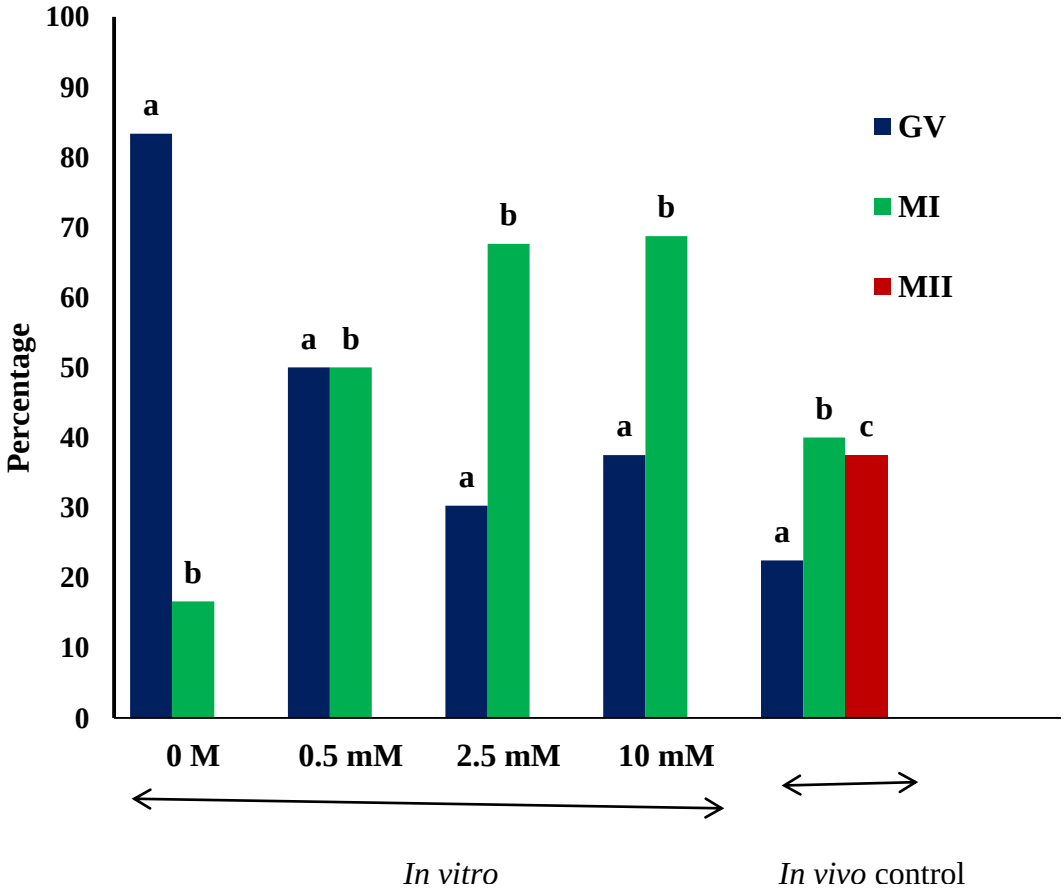


Figure 9: Nuclear maturation of oocytes grown *in vitro* on the basis of nuclear morphology. [a-c]Bar with uncommon superscripts differ significantly ($p<0.05$).

Effects of dbcAMP in maturation of *in vitro* grown buffalo oocytes

In vitro grown oocytes were subjected to do maturation culture. After culture of 24 hours cumulus expansion was observed in a number of oocytes while all oocytes showed cumulus expansion in control group of full-grown oocytes in similar condition (Figure 6 and Figure 7). The typical morphologies of COCs before and after *in vitro* maturation in the medium supplemented with 0, 0.5, 2.5, and 10 mM of dbcAMP in buffaloes are shown in Figures 7 and 8, respectively. In buffaloes, supplementation of the *in vitro* maturation medium with dbcAMP significantly enhanced the rate of cumulus expansion compared to the control group. In vitro grown oocytes mostly remain at GV stage and a small proportion developed to MI stage while fully grown oocytes mature up to MII stage in control (Figure 8, Figure 9 and Table 3).

Table 3. Nuclear maturation of oocytes grown *in vitro*

Oocytes	Concentration of dbcAMP	Number of oocytes examined	Number of oocytes on the basis of nuclear morphology		
			GV	MI	MII
***In vitro* grown**	0 mM	30	25	5	0
	0.5 mM	30	15	15	0
	2.5 mM	33	10	23	0
	10 mM	32	12	22	0
Full-grown oocytes (*in vivo* control)	Control	40	9	16	15

This may be due to inhibition of dbcAMP. Because dbcAMP is known as a meiotic inhibitor. In contrast, Corall *et al.* (1991) reported that dbcAMP improved significantly the ability of oocytes to resume meiosis at the end of the culture period. Their results showed that dbcAMP's ability to inhibit meiosis during culture led to a 7% increase in the proportion of oocytes capable of resuming meiosis by the end of the culture period. However, in the present study, the proportion of oocytes underwent germinal vesicle (GV) breakdown and the number of oocytes reached to MI stage was increased in dbcAMP treated oocytes comparing the oocytes in dbcAMP free media. It has been reported that dbcAMP can improve the ability of growing oocytes as well as grown oocytes to progress into metaphase II by controlling the activity of cytoplasmic factors in oocytes (Sato *et al.,* 1990).

Conclusion

In conclusion, during buffalo oocyte IVM the dbcAMP prevents denudation and degeneration of oocytes and increases survivability in culture by maintaining the meiotic arrest of oocytes and also increases the diameter of oocytes.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research submitted.

Acknowledgements

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