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Theophylline Enhances *In Vitro* Fertilization and Embryo Production in Buffaloes

I. Akter, Md. Hasanur Alam, M. N. Islam, A. K. Modak, A. Khatun, & M. Moniruzzaman*
Department of Animal Science, Bangladesh Agricultural University
Mymensingh-2202, Bangladesh
*Corresponding author: monir.as@bau.edu.bd
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ABSTRACT

This study aimed to investigate the effect of theophylline on *in vitro* fertilization of buffalo oocytes and embryo development in subsequent *in vitro* embryo culture. Cumulus-oocyte complexes (COCs) were collected from large antral follicles of slaughtered buffaloes and matured *in vitro* for 24 hours. *In vitro* matured oocytes were fertilized in Brackett and Oliphant's (BO) media supplemented with four different concentrations of theophylline (0 mM, 2.5 mM, 5 mM, or 10 mM). After 18 hours of *in vitro* fertilization, some of the oocytes or presumptive zygotes were fixed and stained to assess fertilization, while the others were cultured for 7 days to assess their developmental capacity *in vitro*. The results showed that theophylline enhanced the penetration rate of spermatozoa into buffalo oocytes. Supplementation of BO medium with theophylline also increased the normal fertilization rate. In subsequent embryo culture, theophylline increased the formation of 8-cell embryo, morula, and blastocyst rate in buffalo. The cleavage rate did not differ significantly between groups. The morula and blastocyst formation percentages were higher in the groups treated with 2.5 mM theophylline than in the control groups. In conclusion, theophylline improves *in vitro* fertilization rate and embryo production in buffaloes.

Keywords: buffalo; embryo; in vitro fertilization; oocyte; theophylline

INTRODUCTION

The buffalo (*Bubalus bubalis*) is an important component of livestock agriculture in Asian countries. Buffalo milk is highly valued for its high milk fat content (Arora *et al.,* 2022). They are also popular among farmers because of their low maintenance requirements, good feed conversion, utilization of poor-quality roughages, and disease resistance (Phogat *et al.,* 2016). They have low fertility with seasonal anestrous, silent heat, delayed first calving, poor conception rate, and prolonged inter-calving periods (Warriach *et al.,* 2015). To improve and spread their capabilities, assisted reproductive technologies (ARTs) need to be used. Among ARTs, *in vitro* embryo production (IVEP) is an important tool to increase the reproductive performance of buffaloes. IVEP is more limited in buffaloes than in cattle due to the low yield of the blastocyst (Baruselli *et al.,* 2020). The IVEP system consists of three chronological steps-*in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). Among them, *in vitro* fertilization is an essential step for embryo production. IVF can be widely used in the production of farm animal embryos (Mapletoft, 2013). In recent decades, tremendous efforts have been made to improve the success rate of IVF by adding various factors in the media in different animal species. However, the success rates of IVF and IVEP in buffaloes are lower than in the other domestic animal species.

Various factors, such as sperm quality, duration of sperm-egg co-incubation, sperm capacitation mediator, the timing of insemination, environmental conditions, etc., influence the efficiency of IVF. Sperm capacitation is an essential step of successful IVF. Capacitation involves physiological changes in sperm that make them capable of fertilizing the mature oocytes. Freshly ejaculated sperm are not capable of fertilizing the mature oocytes. *In vitro* sperm capacitation must be performed for successful IVF (Okabe, 2018). Several factors are involved in the sperm capacitation process, including Ca^{2+} ionophore A23187 (Tateno *et al.,* 2013), caffeine (Zhang *et al.,* 2022), pentoxifylline (Guasti *et al.,* 2017), relaxin (Ferlin *et al.,* 2012), heparin (Parrish, 2014), D-penicillamine, hypo-taurine, and epinephrine (Kang *et al., 2015)* as well as theophylline (Leahy *et al.,* 2016).

Heparin is commonly used in IVF media as an agent to increase sperm capacity in cattle (Parrish, 2014). Boccia *et al.* (2013) reported that heparin increases the capacitation and fertilization rate in buffaloes. Caffeine improves the progressive motility, straightness, and linearity of sperm movement in porcine sperm (Yamaguchi *et al., 2013*). Theophylline, a member of the methylxanthines, improved cleavage and embryo development rates in cattle (Ferré *et al.,* 2017). Both theophylline and

caffeine act as phosphodiesterase (PDE) inhibitors by increasing the levels of cyclic adenosine monophosphate (cAMP). The increased levels of cAMP help sperm to bind to oocytes that have matured *in vitro* to increase the fertilization rate. Theophylline is considered a more potent PDE inhibitor than caffeine. However, the roles of theophylline in the IVF and embryo production in buffaloes are not yet clear. In the present study, buffalo oocytes were fertilized with theophylline to investigate its effects on IVF and subsequent embryo development *in vitro*.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. All plastic parts were purchased from Corning Incorporated (Corning, NY, USA). Culture grade Millex^{R}-GV syringe filters (0.22 μm of pore size) were procured from Merck Millipore (Tullagreen, Ireland).

Collection and Processing of the Ovaries

The ovaries of buffaloes were collected from a local slaughterhouse at Kaptan Bazaar, Dhaka, and processed as described previously (Islam *et al.,* 2020). Briefly, the ovaries were kept in a thermos flask containing 0.9% physiological saline solution (PSS) and carried to the laboratory. The ovaries were washed 5 times with PSS. Then the ovaries were trimmed to remove the surrounding adipose tissues and stored in PSS until the cumulus-oocyte complexes (COCs) were collected. This study was found ethically sound by Bangladesh Agricultural University Research System (Approval Number: BAURES/ESRC/691/2020).

Collection of the COCs

The buffalo COCs were collected from antral follicles (4–8 mm diameter) with a 10 mL syringe (Henke Sass Wolf, Tuttlingen, Germany) on an 18-gauge needle as described previously (Maksura *et al.,* 2021). Briefly, the aspiration medium consisted of TCM-199 supplemented with 3.2 mg/mL bovine serum albumin (BSA) and 250 μg/mL gentamycin sulfate. The follicular materials (the COCs and follicular fluid) were carefully transferred into a collagen-treated 60-mm disposable Petri dish along with the aspiration medium to allow the COCs to settle. Then, the COCs were collected using a zoom stereomicroscope (CZM 6; Labo America Inc., California, USA). The collected COCs were washed in TCM-199 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, NY, USA), 0.08 mg/mL sodium pyruvate, 1 mM L-glutamine, and 50 μg/mL gentamycin sulfate. The COCs with more than two to three compact layers of cumulus cells were used for *in vitro* maturation.

In Vitro Maturation (IVM)

The *in vitro* maturation medium was TCM-199 supplemented with 10% (v/v) FBS, 5 μg/mL follicle stimulating hormone (FSH; NIDDK, Washington, DC, USA), 0.1 mg/mL sodium pyruvate, 50 μM cysteamine, 1 μg/mL 17β-estradiol, and 0.08 mg/mL gentamycin sulfate. The COCs were placed in 50 μL droplets of maturation medium and coated with paraffin oil. They were cultured in 5% CO_2 in humidified air at 38.5 °C for 24 hours (Maksura *et al.,* 2021).

In Vitro Fertilization (IVF)

The IVF was performed as described previously (Modak *et al.,* 2022). Briefly, frozen semen from a high performing Murrah buffalo bull was collected from the local livestock department. The semen straws were thawed in a water bath at 35 °C for 30 sec. The semen was washed by centrifugation twice at 800 × g for 5 min in modified Brackett and Oliphant's (BO) medium supplemented with 5 mM caffeine and 20 μg/mL heparin. Sperm concentration was 2×10^6 sperm/mL and forward movement was greater than 50%. For capacitation, the sperms were incubated in a basic BO medium without caffeine and heparin in 5% CO_2 in humidified air at 38.5 °C for 4–5 hours. To investigate the effects of theophylline, the BO basic medium was supplemented with 0 mM, 2.5 mM, 5.0 mM, or 10.0 mM theophylline. Finally, the COCs were placed in the sperm droplets (50 μL) and incubated in 5% CO_2 in humidified air at 38.5 °C for 18 hours.

Assessment of Fertilization

After fertilization, some of the COCs (50% of the total COCs) were used for assessment of fertilization and the remaining (50%) for *in vitro* culture (IVC). A fertilization assessment was performed in each IVEP procedure and was done as described by Makita *et al*. (2016). Briefly, the COCs were collected from the IVF medium and denuded in 0.1% (w/v) hyaluronidase using a Pasteur pipette. Zygotes were fixed with aceto-ethanol (1:3; v:v) and stained with aceto-orcein solution (1%; w/v). The nuclear status of zygotes was examined under a differential interference contrast (DIC) microscope (Olympus Corporation, USA). The oocytes that showed two polar bodies and two pronuclei with a sperm tail were classified as normal fertilization. Fertilization other than normal fertilization, such as oocytes with an enlarged sperm head with anaphase/telophase I chromosome, two pronuclei with a sperm tail and one polar body, or more than two sperm heads or pronuclei, were classified as abnormal fertilization.

In Vitro Culture (IVC) of Zygotes

The zygotes were washed three times in pre-warmed Research Vitro Cleave medium (RVCL medium Cook, Australia) supplemented with 1% fatty acid-free BSA. Two-third of the maturation medium was replaced by RVCL medium, supplemented with 10 mg/mL of fatty acid-free BSA, to prepare the medium for IVC of zygotes. The zygotes were cultured at 5% CO_2 in air at 38.5 °C for 7 days. Embryo developments

were examined on days 2, 3, 5, and 7 for cleavage, 8-cell embryo, morula, and blastocyst stages, respectively (Modak *et al*., 2022).

Statistical Analysis

Data were transferred in Microsoft Excel, organized and processed for further analysis. All data were subjected to one-way ANOVA. All data were analyzed by "SAS/STAT version 9.1.3" for Windows Service Pack 4, 2004 SAS Institute, and Cary NC, USA. Differences at $p < 0.05$ were considered statistically significant.

RESULTS

Theophylline Supports Fertilization of Buffalo Oocytes *In Vitro*

The typical morphologies of buffalo oocytes after *in vitro* fertilization are shown in Figure 1. The percentages of oocytes penetrated by sperm were significantly higher when the BO medium was supplemented with 2.5 mM theophylline in the groups than with 0 and 10 mM theophylline (Table 1). Similarly, the supplementation of BO medium with 2.5 mM theophylline significantly increased the fertilization rate of oocytes compared with the groups with 0 mM and 10 mM theophylline (Table 1). However, the percentage of abnormal fertilization rates did not differ between the theophylline supplemented groups. These findings suggest that theophylline supplementation improved sperm penetration and the fertilization rate of buffalo oocytes *in vitro*.

Theophylline Improves Development of Buffalo Embryos *in Vitro*

To evaluate developmental ability, the zygotes were further cultured for 7 days. Representative morphologies of buffalo embryos are shown in Figure 2. The cleavage stages, 8-cell embryo, morula, and blastocyst stages were examined on the second, third, fifth, and seventh days of the culture period. The supplementation of theophylline to the IVF medium positively affected the development of the buffalo embryos (Table 2). Cleavage rates were significantly higher ($p<0.05$) at 2.5 mM theophylline concentrations than at 10 mM. There were no significant differences in the cleavage rates of embryos between treatment groups (Table 2). The percentage of 8-cell embryos was higher in the group supplemented with 2.5 mM theophylline than in the groups supplemented with 0 mM and 10 mM groups. Theophylline at a concentration of 2.5 mM promoted embryo development at the morula stage significantly more than the other theophylline concentrations. Similarly, the percentage of embryos that developed into blastocysts was higher in 2.5 mM theophylline

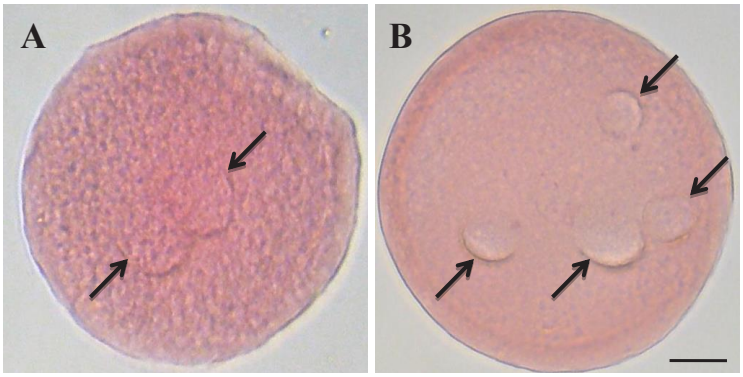

Figure 1. Morphologies of buffalo oocytes after *in vitro* fertilization. Oocytes were incubated in Brackett and Oliphant's (BO) medium supplemented with 0 mM, 2.5 mM, 5 mM, and 10 mM theophylline for 18 hrs. The oocytes were then fixed and stained with aceto-orcine for fertilization assessment. Zygotes were classified as normally fertilization (A; zygote with one female and one male pronuclei) and abnormal fertilization (B; zygote with more than two pronuclei). Scale bars represent 20 μm.

Table 1. *In vitro* fertilization of buffalo oocytes treated with theophylline

Concentrations of theophylline (mM)	Number of matured oocytes	Number (%) of oocytes		
		Penetration	Fertilization	Abnormal fertilization
0	25	10 (39)^{b}	8 (31)^{bc}	2 (8)
2.5	30	21 (70)^{a}	18 (60)^{a}	3 (10)
5	25	14 (55)^{ab}	12 (48)^{ab}	2 (8)
10	20	4 (18)^{c}	3 (15)^{c}	1 (5)

Note: Matured COCs were subjected to *in vitro* fertilization with different concentrations (0 mM, 2.5 mM, 5 mM, and 10 mM) of theophylline. Normal fertilization: the oocytes that have two pronuclei and two polar bodies. Abnormal fertilization: fertilization other than normal fertilization; for example, the oocytes have an enlarged sperm head with anaphase/telophase I chromosomes, two pronuclei with a sperm tail and one polar body, or more than two sperm heads or pronuclei (polyspermy). Data are shown in total number (%) of oocytes from at least three replicated cultures.
$^{a\text{-}c}$Means in the same column with different superscripts differ significantly ($p<0.05$).

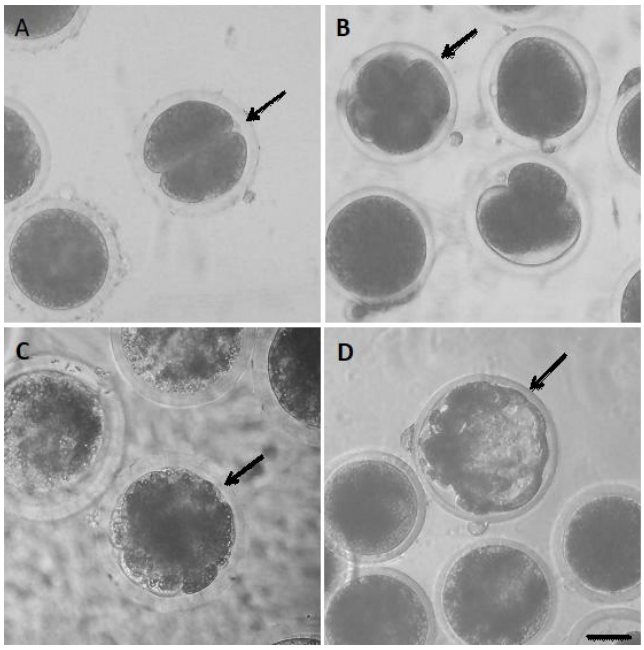

Figure 2. Representative morphologies of buffalo embryo developed *in vitro* over 7 days. Arrows indicate cleavage (A), 8-cell (B), morula (C), and blastocyst (D) stages at days 2, 3, 5, and 7. Scale bars represent 60 mm.

Table 2. *In vitro* development of buffalo embryo treated with theophylline

Concentrations of theophylline (mM)	Numbers of oocytes examined	Numbers (%) of embryo			
		Cleaved	8 Cell	Morula	Blastocyst
0	35	11 (31.6)^{ab}	6 (16.8)^{bc}	4 (11.5)^{b}	1 (2.8)^{b}
2.5	35	18 (51.1)^{a}	15 (42.2)^{a}	13 (36.7)^{a}	5 (14.4)^{a}
5	33	14 (42.8)^{ab}	8 (24.4)^{ab}	6 (17.7)^{b}	2 (5.9)^{ab}
10	28	3 (10.8)^{b}	1 (3.3)^{c}	0	0

Note: Zygotes were cultured for embryo developments. The embryonic development were examined at day 2, 3, 5, and 7 for cleavage, 8-cell embryo, morula and blastocyst stages, respectively. Data are shown in total number (%) of oocytes from at least three replicated cultures. a-cMeans in the same column with different superscripts differ significantly ($p<0.05$).

group than control (without theophylline). Therefore, supplementation of IVF medium with theophylline promoted the development of 8-cell embryo, morula, and blastocyst *in vitro*. However, the developmental competence of the embryo was reduced at a concentration of 5 mM theophylline, and none of the fertilized zygotes developed to morula and blastocyst at a concentration of 10 mM theophylline.

DISCUSSION

Fertilization in mammals depends on a series of processes that occur in both the oocyte and the sperm before and after their fusion. Before fertilization, the oocytes extend their polar bodies forward and prepare for fertilization. On the other hand, the sperm become hyperactivated and are ready to fertilize the oocytes. The penetration of hyperactivated and capacitated sperm into an oocyte is required to complete meiosis and fusion between male and female germ cells. In the present study, sperm incubated in BO medium supplemented with theophylline increased the penetration rate of sperm into the oocytes. This result indicated that theophylline increased the penetrating capacity of sperm. Theophylline increased sperm motility and penetration capacity (Kang *et al.*, 2015). Theophylline proved to be a harmless, effective agent for reviving immotile sperm in problems with retrograde ejaculation in humans (Ebner *et al., 2014)*. Theophylline is a methylxanthine and has three types of cellular mechanisms- intercellular calcium shift, inhibition of phosphodiesterase to prevent cAMP breakdown, and blockade of adenosine receptor. Hyperactivation of hamster sperm was inhibited by Ca^{2+} channel blocker (Stauss *et al.*, 1995). Subsequently, sperm cation channel (CatSper) null mice were developed. Sperm from that nulls failed to hyperactivate and enter zona pellucida (Luque *et al.*, 2021). Similarly, incubation of boar spermatozoa with a cell-permeable cAMP analog (cBiMPS) was found to activate transient receptor potential cation channel subfamily C member 3 (TRPC3), which induce hyperactivation (Otsuka & Harayama, 2017). Based on these observations, in the present study, it was hypothesized that theophylline increased Ca^{2+} and cAMP concentrations in the sperm, inducing hyperactivation and penetration capacity of buffalo sperm.

During *in vitro* fertilization, a significant increase in the normal fertilization rate between oocytes and sperm

was observed in a medium supplemented with theophylline. This result indicates that theophylline promotes normal *in vitro* fertilization in buffaloes. Theophylline has been shown to accelerate the process of fertilization *in vitro* (Ferré *et al.,* 2015). Theophylline preserves the motility of frozen sperms and even sperms collected in testicular biopsy (Gorji *et al.,* 2018). Theophylline efficiently increases the formation of male pronuclei from porcine sperm under culture conditions (Yoshioka *et al.,* 2003). Since theophylline induced the formation of male pronucleus (Yoshioka *et al.,* 2003) but had no effect on oocytes during fertilization (Takahashi & First, 1993), it could be concluded that theophylline efficiently produced male pronuclei in our culture system, which in turn increased normal fertilization rate of buffalo oocytes *in vitro*. Our results showed that the development of compact morula and blastocyst increased under theophylline-supplemented culture condition. Cleaved embryos from abnormal fertilization are unable to develop to the blastocyst stage (Fragouli *et al., 2014).* This could be a reason for the increased embryo production in buffalo oocytes treated with theophylline in our experiment. Thus, theophylline has a positive effect on blastocyst yield *in vitro*.

In the present study, fertilization and embryonic development were reduced by a higher dose of theophylline (10 mM). At this concentration, a low cleavage rate and 8-cellls that did not develop further were produced. This suggests that higher theophylline concentration disrupts the sperm capacitation process, reducing fertilization and embryonic development *in vitro*. Ibis *et al*. (2021) suggested that a higher dose of theophylline decreases ATP production, which reduces sperm motility and fertilization. A theophylline concentration of 10 mM alters sperm respiration and glycolysis, leading to intracellular acidification, which ultimately suppresses the capacitation process (Parrish *et al.,* 1989). In the present study, a high concentration of theophylline also negatively affects sperm penetration rate, fertilization rate, and further development of the buffalo embryo. Yoshioka *et al*. (2003) found that a higher concentration of theophylline reduced the number of sperm. Methylxanthines, including theophylline induced structural and functional alterations in embryos, caused embryotoxicity and teratogenicity (Basnet *et al.,* 2017). This could be a reason for the lower fertilization rate and further development of buffalo embryos in a higher concentration of theophylline in the present study. However, our study lacks molecular procedures that might predict the working procedure of theophylline. The further molecular study is needed to reveal the cellular signaling cascade of theophylline on IVF and embryo production in buffaloes.

CONCLUSION

Theophylline at a concentration of 2.5 mM accelerates sperm penetration rate, fertilization rate, and improves the efficiency of subsequent *in vitro* embryo production in buffalo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could affect the impartiality of the research report.

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