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In vitro Maturation and Fertilization of Buffalo Oocytes Cultured in Media Supplemented with Bovine Serum Albumin

Research Article

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ABSTRACT

The aim of this study was to determine the quality of cumulus-oocyte-complexes (COCs) and the effects of bovine serum albumin (BSA) supplementation on *in vitro* maturation and fertilization rate of buffalo oocytes. COCs were collected from slaughterhouse buffalo ovaries by aspiration method. Only normal grades COCs were matured for 48 hours in TCM-199 media. Two groups were created: one for the maturation medium supplemented with 5% of BSA, the other without supplementation (control). Matured oocyte fertilized with capacitated frozen-thawed semen in Brackett and Oliphant (BO) medium for 5 hours, in an incubator at 38.5 °C with 5% CO_2 under humidified air. A significantly higher number of normal quality COCs per ovary ($P<0.05$) were obtained from ovaries devoid of *corpus luteum* (CL) compared to ovaries having CL (1.84 *vs.* 0.81) respectively. The percentage of oocytes reaching Metaphase-II (M-II) stages was 58.07±2.08 and 68.10±0.75% for control and 5% level of BSA respectively. The fertility level was assessed by pronuclei formation: the normal fertilization rate (2PN) obtained was 19.63±3.11 and 29.52±1.98% for control and BSA supplementation respectively. Significant differences ($P<0.05$) were observed in maturation (M-II) and fertilization (2PN) rate of buffalo oocyte by adding 5% level of BSA supplementation in culture media. Thus, data gathered in this study showed that 5% BSA supplementation in both maturation and fertilization media can be used for enhance the maturation and fertilization rate of buffalo oocytes, as well as to improve the grade of collected buffalo COCs.

KEY WORDS BSA, buffalo, cumulus-oocyte-complexes, IVF, IVM.

INTRODUCTION

Buffalo is an economically important livestock species in Bangladesh, like in many south Asian countries. The total buffalo population is about 1.39 million in Bangladesh (BER, 2012), with about 40% of the population in the coastal regions (Faruque *et al.* 1990). The livestock sector of Bangladesh foresees its genetic improvement, mainly regarding the reproductive performance and quality of meat and milk. Obviously, successful buffalo breeding highly depends on the genetic improvement. Buffaloes are reported to have low reproductive performance with several inherent reproductive problems, such as silent estrus, seasonal anestrous, delayed puberty, delayed first calving, late post partum conception and a long calving interval (Nandi *et al.* 2002). In order to improve reproductive efficiency of buffalo, assisted reproductive technologies such as artificial insemination (AI), multiple ovulation and embryo transfer

(MOET) and *in vitro* production of embryos have been introduced (Nandi *et al.* 2002). Application of these technologies in assisted reproduction of buffalo is necessary to rescue the precious germplasm due to wastage by indiscriminate slaughter of this animal. These techniques help to improve the productive and reproductive potential of the buffalo population.

In vitro embryo production (IVEP) could be an effective technique to improve efficacy of transferable embryo production (Drost, 2007).

However, substantial improvement to the *in vitro* embryo production systems available for buffalos is required, which still remain in sub-optimal levels, compared to those applied in bovines.

Therefore, considerable basic developmental work still has to be undertaken in order to standardize IVEP techniques for buffalos. An critical goal for mass production of buffalo embryos is the recovery of a large number of oocytes with high developmental competence. Oocyte quality is one of the major factors determining the success of *in vitro* embryo production (Krisher, 2004). In addition, proper oocyte selection at the laboratory is crucial for successful embryo production. Another critical step regards the *in vitro* maturation.

The culture technique employed in IVM not only affects the proportion of bovine oocytes that reach metaphase II (M II) and become capable of undergoing *in vitro* fertilization, but can also influence the subsequent embryonic development (Bavister *et al*. 1992).

Galli and Lazzari (2003) and Neglia *et al.* (2003) refer to the complexity of fertilization and lower cleavage rate in buffalo.

It is of upmost importance for a successful *in vitro* production (IVP) of buffalo embryos the evaluation of ovaries, and an efficient collection and grading of oocytes. Often, IVF of buffalo oocyte is performed using fresh semen or in ordinary manner, but frozen semen and the optimization of the maturation medium can contribute for the success of *in vitro* production of buffalo embryos.

In Bangladesh, IVP of buffalo embryos is a new concept. Although, some work has been practiced in IVP of embryos over different species such as cattle, goat and mouse few experiments exist on the collection, evaluation and grading of COCs from slaughterhouse buffalo ovaries (Khandoker *et al.* 2011), or using species hybridization through fertilization with cattle spermatozoa (Khandoker *et al.* 2012). From this point of view, the present study was undertaken aiming to collect and evaluate buffalo´ ovaries, follicles and COCs obtained at the slaughterhouse; additionally it was also intent to analyze the effects of BSA supplementation in the maturation and fertilization media in the *in vitro* maturation and the fertilization rate of buffalo oocytes.

MATERIALS AND METHODS

Collection and processing of ovaries

Buffalo ovaries were obtained from a slaughterhouse, were placed in normal saline (0.9% NaCl) and transported to the laboratory in a thermo flask maintained at 25 °C to 30 °C within 5 to 6 hours. The ovaries were rinsed thoroughly, twice, in physiological saline solution at 25 °C. In the laboratory, each ovary was isolated from the surrounding tissues and overlying bursa, and washed three times in D-PBS, and then twice in oocyte harvesting medium (D-PBS+4 mg/mL BSA+1.50 IU/mL Penicillin) as described by Wani *et al.* (2000). After collection and trimming, ovaries were evaluated on the basis of presence and absence of CL.

Collection and evaluation of oocytes

Buffalo oocytes were aspirated (as described by Wani *et al.* 2000) using a 18 G needle coupled to a 10 mL syringe filled with D-PBS (1.0-1.5 mL); all 2-6 mm diameter follicles were aspirated. The follicular content was then transferred to a 90 mm Petri dish, slowly to avoid damaging the cumulus cells. The total number of oocytes harvested was counted under a stereomicroscope. COCs were classified into 4 grades as described by Khandoker *et al.* (2001), according to the morphology of the cumulus cells and nucleus. Briefly, in Grade A the oocytes completely surrounded by cumulus cells; in Grade B the oocytes partially surrounded by cumulus cells; Grade C oocytes are not surrounded by cumulus cells; and in Grade D, degeneration was observed in both the oocytes and the cumulus cells. Grades A and B were considered as normal and grade C and D as abnormal COCs.

Evaluation of *in vitro* nuclear maturation

For *in vitro* maturation of COCs, two different culture media were used: one using 5% BSA supplementation and the other without it. Unless otherwise mentioned, all chemicals and media were purchased from Sigma Chemical co. (St. Louis, MO, USA). The IVM of oocytes was performed using a medium consisting of bicarbonate-buffered tissue culture medium (TCM-199), 0.22 m*M* sodium pyruvate, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. To study the effects of bovine serum albumin on the maturation rate of buffalo oocytes, to this medium 5% BSA was added in the tested BSA supplemented medium. The pH was adjusted to 7.3-7.4 in all the media and they were filtrated through a 20 µm sartorius Minisart filter (Toyo Roshi Co. Ltd., Japan). From each group of maturation media, about 1-4 drops of 100 µL were prepared into each of two 35 mm culture dishes. Batches of 13-15 oocytes were then transferred into these, covered with paraffin oil (Loba Chemic Pvt. Ltd., India) and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2, for 48 hours. The level of

nuclear maturation was then assessed in each dish. For this purpose, 25% of cultured COCs were randomly sampled from each droplet. COCs were then denuded and mounted on glass slide and fixed with ethanol:acetic acid (3:1).

After fixation, oocytes were stained with 1% (w/v) orcein in acetic acid for 10 min, and rinsed with glycerol: acetic acid: water (1:1:3). The nuclear stage was evaluated under a phase-contrast microscope (Olympus, Tokyo, Japan). Oocytes displaying a metaphase plate and one pink color polar body (PB) were regarded as being at MII stage. The nuclear maturation rate in this study was evaluated in 3 replicates, in a total of 85 oocytes.

Semen collection and preparation of frozen semen

Semen was collected by artificial vagina (AV) from buffalo in the Bangladesh agricultural university dairy farm, Mymensingh. After semen assessment, semen was diluted in the Triladyl based cryodiluent (diluter+cryoprotectant) in a final concentration of 2×10^6 spermatozoa per mL. The motility of the equilibrated sperm was checked and only samples with more than 60-70% motility were used for freezing, as described by Apu *et al.* (2012). The frozen semen straws were transferred into the liquid nitrogen container, at -196 °C, until use.

Insemination and checking the fertilization rate

The Brackett and Oliphant (BO) fertilization medium at pH 7.8, prepared on the day of use, was supplemented with 1% penicillin, 10µg/mL heparin and 2.5 m*M* caffeine, while 1% BSA supplementation was added in one of the media. One to four insemination droplets (100 µL) of BO medium depending on the number of the matured COCs were prepared for sperm capacitation in a 35 mm culture dish, covered with paraffin oil and were kept in the incubator for 5-6 hours. The frozen semen straws were retrieved from the liquid nitrogen (LN_2) container immediately prior to use and thawed in warm water at 37 °C for 12 seconds.

After 48 hours of incubation, mature COCs were used for fertilization. Two 35 mm culture dishes filled with washing solution (BO+1% BSA) were used to wash the COCs 3 times. About 14-16 COCs with minimum volume of medium were then transferred to each of the sperm drops previously prepared, and cultured for 5 hours in a humidified CO_2 incubator at 38.5 °C. Afterwards, all the COCs from each drop were denuded by repeated pipetting, fixed in a glass slide with aceto-ethanol (acetic acid: ethanol, 1:3, v/v) and stained with 1% aceto-orcein, to assess fertilization. The slides were examined at high magnification (100X) to score pronuclei (PN) formation as follows: oocyte with two PN–Normal fertilization, oocyte with one PN–Asynchronous PN development / parthenogenetic activation or one PN was obscured by lipid droplets and oocyte with more than two PN–Polyspermia. The fertilization rate in this study was calculated on the basis of pronuclei formation; a total of 85 oocytes were tested in 3 replicates.

Statistical analysis

Data generated in this experiment were entered in Microsoft Excel worksheet, organized and processed for further analysis. The analysis of variance (ANOVA) was used to compare the oocyte recovery rates, while the effects of CL presence on oocyte recovery were analyzed using Student's t-test. For *in vitro* maturation and fertilization, the values analyzed by ANOVA and followed by a least square difference (LSD) test, using SPSS software version 16.0 (SPSS inc, Chicago, IL, USA).

RESULTS AND DISCUSSION

Ovarian category and COCs collection per ovary

A total of 134 buffalo ovaries (3 replicates) were obtained from the slaughterhouse and categorized into 2 groups based on presence (n=49) or absence (n=85) of *corpus luteum*. A total of 828 follicles were counted at the ovarian surface, 608 being from CL absent and 220 from CL-containing ovaries. Not all these follicles were suitable for aspiration. The total number of aspirated follicles was 624, from which 446 were aspirated from CL-absent ovaries, while 178 were aspirated from ovaries presenting CL. A significantly higher ($P<0.05$) number of follicles was observed in CL-absent ovaries than in CL-containing types (Table 1). Consequently, the number of aspirated follicles from CL-absent ovaries was significantly higher ($P<0.05$) than those aspirated from CL-containing ovaries (Table 1). Significantly higher ($P<0.05$) number of normal COCs was found in CL-absent ovaries compared to CL-containing ovaries.

The mean retrieved COCs per ovary was higher in CL-absent ovaries than in CL-present ovaries (2.76 *vs.* 1.73 COCs per ovary, respectively). The presence of CL in cyclic female's ovary leads to an increased level of progesterone that signals a negative response to anterior pituitary gland for the restriction of gonadotrophin secretion and ultimately follicular degeneration occurs (Webb *et al.* 1999). The cause for the low number of oocytes in ovaries presenting a CL is likely because of the restricted follicular development, as lutein cells occupy a great portion of the ovary; furthermore, CL may inhibit the follicular growth and foster their atresia (Hafez, 1993). In this study, the total number of follicles (7.15±0.16), aspirated follicles (5.24±0.15), normal COCs (1.84±0.08) and total number of COCs (2.76±0.10) per ovary were significantly higher ($P<0.05$) in ovaries without corpus luteum, including ovaries from non-cycling females.

Table 1 Types and number of follicles and cumulus-oocyte-complexes (COCs) per ovary

Types of ovaries	Total no. of ovaries	Follicles per ovary, Mean ±SE (Total no. of follicles)	Aspirated follicles per ovary, Mean ±SE (Total no.)	Collected COCs per ovary Mean ±SE, (Total no.)		COCs per ovary, Mean ± SE, (Total no.)
				Normal	Abnormal	
CL absent ovary	85	7.15 [a] ±0.16 (608)	5.24 [a] ±0.15 (446)	1.84[a] ±0.08 (157)	0.91[a] ±0.06 (78)	2.76[a] ±0.10 (235)
CL present ovary	49	4.48 [b] ±0.24 (220)	3.63 [b] ±0.17 (178)	0.81 [b] ±0.05 (40)	0.93[b] ±0.09 (46)	1.73[b] ±0.09 (86)
Total	134	6.17±0.17 (828)	4.65±0.13 (624)	1.47±0.07 (197)	0.92±0.05 (124)	2.39±0.08 (321)

Values in the same column with different superscripts are significantly different (P<0.05).

The buffalo destined slaughtering are usually females with lower reproductive performance and most of them might be acyclic. So, a random sampling at the slaughterhouse showed an increased possibility to obtain more non-cyclic ovaries than cyclic ones. The ovary with less number of CL obtained in this experiment supports the above statement. It was found that the presence of a CL significantly reduced the recovery rate as well as the quality of the oocytes. Which support the earlier reports in buffalo (Singh *et al*. 2001; Jamil *et al.* 2008; Hoque *et al.* 2011; Sahoo *et al.* 2013). However, the mean oocyte recovery in buffalo is lower when compared with cattle (Wang *et al.* 2007). This difference may be attributed to the considerably lower primordial follicles reserve in buffalo ovaries than in cattle. The higher number of COCs in CL-absent ovaries compared to that of CL-present group found in the present study may be explained by the role of hormonal balance (FSH and LH) on buffalo folliculogenesis. The negative effect of progesterone might not be fully functional and estrogen-progesterone remains in balanced levels, which allows the follicular growth and oocytes maturation. This results support the previous report of Khandoker *et al.* (2011) on evaluation of buffalo ovaries, and those of Amer *et al.* (2008) who reported a significantly greater (P<0.01) number of 2-6 mm follicles in CL-absent ovaries.

Effect of supplementation on *in vitro* maturation (IVM) of buffalo oocytes

In this study, the collected COCs were matured in TCM-199 medium supplemented with 5% bovine serum albumin (BSA) to find out the effect of supplementation on *in vitro* maturation of buffalo oocytes, as confirmed by nuclear maturation. The percentage of matured oocytes reaching the M-II stage was significantly higher (P<0.05) in 5% BSA than in the control media (68.10±0.75 and 58.07±2.08 respectively. The percentage of follicles in M-I stage was 16.95 ± 0.73 and 14.12 ± 0.37 respectively for control and BSA media. The percentage of germinal vesicle breakdown was 7.03 ± 2.29 and 5.01 ± 0.85 in control and BSA media respectively; the germinal vesicle was present in 16.71% ± 2.59 and 14.09% ± 0.93 of the oocytes in control and 5% BSA media respectively (Table 2) and 14.09% ± 0.93 of the control and 5% BSA media respectively (Table 2). The maturation rate in this study was comparable to that reported by Hammam *et al.* (2010), who obtained 68.0 ± 2.4% maturation rate for TCM-199 supplemented with BSA, and to that of Wang *et al.* (2007) in goats, thus suggesting that the maturation rate does not depend on the collection technique. Oocyte maturation might be assessed through the degree of cumulus cell expansion. Farin *et al.* (2001) refer that the bovine serum albumin serves as a protein source and may have hormones bound to it. Therefore, BSA is considered semi-defined but it is likely to contain fewer uncharacterized factors. The cumulus cells surrounding the oocyte play an essential role in promoting oocyte maturation, and they are known to supply nutrients, energy substrates and to mediate the positive effects of hormones on the COCs (Krisher, 2004). Proper maturation is essential for an oocyte to achieve full developmental competence for fertilization.

It seems that BSA contains a number of known growth factors playing an important role in the regulation of oocyte maturation. Via cumulus cells it also prevents the hardening of zona pellucida. Moreover, the beneficial action of BSA may be related to its anti-oxidant properties, which favors the increased maturation rate (Mahmoud and Nawito, 2003). Cumulus cell expansion during *in vitro* oocyte maturation was advantageous for completion of the maturation process.

The role of the cumulus cells might derive from their ability to produce pyruvate to provide energy substrate during the maturation process, as reported by Ball *et al.* (1984). Obviously, the success of fertilization depends on the nuclear maturation paralleling the cytoplasmic maturation for completion of meiotic division.

Therefore, the *in vitro* maturation process is supposed to be completed when the highest percentage of M-II oocytes is observed. Moreover, the culture condition in 5% BSA supplementation medium as used in the present experiment might be suitable for an increase in the maturation rate of buffalo oocytes.

Table 2 Effects of BSA on *in vitro* maturation of buffalo oocytes

Treatment	Total number of COCs	Rate of Nuclear Maturation Mean% ± SEM, (N)			
		M II	M I	GVBD	GV
Control	41	58.07^{b}±2.08 (24/41)	16.95^{a}±0.73 (7/41)	7.03^{a}±2.29 (3/41)	16.71^{a}±2.59 (7/41)
BSA (5%)	44	68.10^{a}±0.75 (30/44)	14.12^{a} ±0.37 (6/44)	5.01^{a}±0.85 (2/44)	14.09^{a}±0.93 (6/44)

Values in the same column with different superscripts are significantly different (p<0.05).
M II: metaphase-II stage; M-I: metaphase-I stage; GVBD: germinal vesicle breakdown stage; GV: germinal vesicle present.

Table 3 Effects of BSA on *in vitro* fertilization of buffalo oocyte

Treatment	Total number of COCs	Rate of fertilization mean% ± SEM, (N)			
		2 PN	1 PN	3 PN	No PN
Control	41	19.63^{b}±3.11(8/41)	7.48^{a}±2.38(3/41)	4.96^{a}±2.63(2/41)	68.19^{a}±4.94 (28/41)
BSA (5%)	44	29.52^{a}±1.98(13/44)	9.21^{a}±2.54(4/44)	6.82^{a}±0.16(3/44)	54.44^{b}±2.93 (24/44)

Values in the same column with different superscripts are significantly different (P<0.05).
2 PN: normal fertilization; 1 PN: no fertilization; 3 PN: oocyte with polyspermia.

In vitro fertilization (IVF) of buffalo COCs with buffalo frozen semen

After oocytes maturation in TCM-199 supplemented with BSA, about 29.52% of the oocytes were successfully fertilized (2PN) with buffalo frozen-thawed semen. The pronuclear formation (PN) rates is summarized in (Table 3). The percentage of oocytes forming 1PN, 2PN or 3PN were 9.21 ± 2.54, 29.52 ± 1.98 and 6.82 ± 0.16% respectively, while 54.44% ± 2.93 of the oocytes in 5% BSA supplemented medium did not show any PN. In non-supplemented medium (control), the percentage of oocytes forming 1PN, 2PN and 3PN were 7.48 ± 2.38, 19.63 ± 3.11 and 4.96 ± 2.63% respectively, whereas 68.19 ± 4.94 % of the oocytes did not show any PN. It was observed that a significantly (P<0.05) higher percentage of normal fertilization (2PN) occurred in supplemented (29.52%) compared with control (19.63%) groups.

IVF is the most critical step of the IVEP procedures in buffalo; lower cleavage rates than those obtained in other domestic species have been widely reported (Neglia *et al.* 2003; Gasparrini *et al.* 2004; Kumar *et al.* 2007; Anand *et al.* 2008). The medium type and the addition of granulosa cells significantly affect the fertilization rates, as reported by Fuki *et al.* (1989). Neglia *et al.* (2003) reported a lower blastocyst yield in buffalo compared to cattle (26 vs 34 %, respectively). *In vitro* fertilization rate depends on several factors like the maturation medium, the fertilization medium, the semen quality and many other things. The spermatozoa in this experiment were treated with the appropriate concentration of heparin to induce capacitation and subsequent acrosome reaction. Also, the media used for IVF was the BO, which contains motility enhancing substances like caffeine. During IVF, caffeine act as cyclic nucleotide phosphodiesterase inhibitor, and has been used as a motility-stimulating agent. The fertilization rates in this study were significantly different (P<0.05) between supplemented (5% BSA) and control group.

It was showed in the current study that the presence of CL significantly reduced the oocyte yield but do not affected the *in vitro* oocyte development, which supports the previous statement of Mahesh *et al.* (2014).

Adding BSA to the fertilization medium will promote destabilization of the cell membranes of oocytes and sperm by removing cholesterol and zinc molecules which eventually enhances capacitation and acrosome reaction.

Hammam *et al.* (2010) found 15.5% fertilization rate with BO medium, a lower rate than the obtained in the present study. This may be due to the combined effects of successful matured oocytes, cultured condition and semen quality used in this experiment. The fertilization rate (29.52%) observed in this study was comparable with that reported by Totey *et al.* (1992), who found 29.8% fertilization rate of buffalo oocyte with frozen thawed buffalo spermatozoa prepared in BO medium. Although the comparison of fresh and frozen semen effects was not considered in this study, the type of semen used might have influenced the fertilization rate. Seaton *et al.* (1991) reported that fresh sperm gave better penetration rates than frozen-thawed sperm. Frozen-thawed semen is likely to deteriorate more rapidly than fresh ones (Gordon, 1994). Differences in the fertilization rate were tentatively explained by Sirard and Lambert (1985) and by Stubbings and Woski (1991), by individual bull differences in the sperm ability to oocyte penetration.

Moreover, Bracket and Oliphant (1975) indicated that individual variations are also present in the fertilizing ability of the ova. The cumulus cells and their associated matrix were present in this experiment and they might also influence the fertilization rate in a non-specific manner by just increasing the contact area between the spermatozoa and the oocytes. Considering the fertilization rate of buffalo oocytes using frozen-thawed buffalo semen, the supplementation of BSA on fertilization media increased the fertilization rate of buffalo oocytes compared to non-supplemented control media. So, there is a great flexibility of using sup-

plementation in fertilization media and frozen semen for increase the fertilization rate of buffalo oocytes *in vitro.*

CONCLUSION

The present study focused on the influence of the presence or absence of CL on quantity, quality and developmental ability of oocyte from buffalo ovaries, as well as on the BSA supplementation in IVM and IVF of buffalo oocytes. After the above discussion, we could conclude that the oocyte yield per ovary was higher in ovaries without *corpus luteum* and that normal grade COCs (Grade A and Grade B) were suitable for *in vitro* production (IVP) of buffalo embryos. Considering the effects of BSA on the *in vitro* maturation and fertilization of buffalo oocyte, 5% BSA can be advantageous as a supplement of maturation and fertilization media to increase the developmental rate of buffalo oocyte. Moreover, this result creates a great opportunity of conducting further research on buffalo embryo production in Bangladesh.

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