Normal and Abnormal Fertilisation of Zebu Cattle Oocytes *In Vitro*

Anup Kumar Talukder[1], Mohammed Shamsuddin[1,*], Mohammad Bozlur Rahman[1], Farida Yeasmin Bari[1] and John J Parish[2]

[1]*Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh*
[2]*Department of Animal Sciences, University of Wisconsin-Madison, Madison WI 53706, USA*

ABSTRACT

Successful *in vitro* embryo production heavily relies on the normal maturation and fertilisation of oocytes. We examined the normal and abnormal fertilisation of zebu cattle oocytes matured *in vitro*. Immature cumulus oocyte complexes (COCs) from zebu cattle ovaries at slaughter were matured *in vitro* (IVM) for 24 h. The oocytes were either fixed, stained and examined for nuclear changes or fertilised *in vitro* (IVF) with Percoll-separated, heparin-treated spermatozoa (1.0×10^6/mL) of zebu ($n = 7$) and crossbred bulls ($n = 7$). After 18 h of sperm-COCs co-incubation at 39℃ with 5% CO_2 in humidified air, the presumptive zygotes were fixed, stained and examined for pronuclei. The number of oocytes retrieved per ovary was 5.4 ± 0.7. The percentage of matured oocytes was 73.0. The difference in motility of spermatozoa before and after Percoll seperation was significant ($p<0.001$). The percentages of normal and abnormal fertilisation (polyspermia and oocytes with one pronucleus) varied significantly depending on individual bulls ($p<0.05$). A protocol for IVF of IVM oocytes in Bangladeshi zebu cattle is developed. A future study may elucidate the capacity of such IVM-IVF oocytes to develop to the blastocyst stage for transfer to surrogate mother.

(Key words : *in vitro*, maturation, fertilisation, zebu cattle)

INTRODUCTION

In vitro fertilisation, an assisted reproductive technology, has been used extensively in embryo research in many countries since the late eighties but IVF protocol for zebu cattle embryo production is still under research. In fact, IVF has a great prospect in developing the dairy industries of tropical countries. Milk production in tropically adapted non-descript zebu (*Bos indicus*) cows of Bangladesh is low compared with that of exotic (*Bos taurus*) one. The first crossbred (F_1) cows derived from zebu females inseminated with semen from exotic bulls show a better adaptability and produced more milk at tropics (Syrstad, 1996; Alam, 2001). In conventional artificial insemination (AI) technique, breeding an F_1 cow to produce an F_1 calf is not possible. That means AI technique has great limitation in maintaining F_1 cows, which have proved to produce more milk. To address this issue, IVF could be an effective tool to bred crossbred cows with genetically known F_1 embryos. Further, to do AI with progeny tested imported semen is expensive for smallholder dairy farmers. In this regard, IVF could be another alternative approach in producing large number of embryos using single AI dose semen.

In Bangladesh, first attempt was taken to establish a protocol for *in vitro* maturation, fertilisation and culture of zebu cattle oocytes in 2005 and reported that the *in vitro* produced zygotes did not develop beyond the 8-cell stages of embryos (Das, 2005). This indicates that, there might be problems in oocyte maturation, or in fertilisation or in early embryo development or in all three stages of IVF protocol. A follow up (second) experiment on IVF in Bangladesh was done; however, a few of such embryos were developed up to the morula stage (Islam *et al.*, 2007). But *in vitro* development of embryos from zebu cattle oocytes up to transferable blastocyst is not yet developed in Bangladesh. This indicates that, the protocol needs looking into the details of oocyte maturation and fertilisation *in vitro*. Therefore, the aim of the present study was to examine the maturation of oocytes in Bangladeshi zebu cattle and normal and abnormal fertilisation of such oocytes *in vitro*.

MATERIALS AND METHODS

Unless otherwise mentioned, all the chemicals, reagents, me-

† This study was supported by the grant (BG-ARS-121) of the United States of Department of Agriculture (USDA), USA.
* Correspondence : E-mail : m.shamsuddin@gmail.com

dia, biologics and media constituents were purchased from Sigma-Aldrich Chemicals, St Louis, USA. Media and reagents were prepared under standard protocol following aseptic technique (Parrish *et al.*, 1988). The final media for maturation and fertilisation were filtered (0.22 μm poor size, Durapure® membrane filter, Ireland) and routinely equilibrated at 39℃ with 5% CO_2 in humidified air for at least 2 h before use. Only cell culture tested chemicals were used to formulate the media.

1. Aspiration of COC_s and *In Vitro* Maturation

Ovaries of cattle were obtained at slaughter and transported to the laboratory in a thermo flask containing warm saline (35℃, 0.9% sodium chloride solution, w/v), supplemented with penicillin-streptomycin (125 μg/ml Streptopen®, Renata Bangladesh Ltd., Dhaka, Bangladesh) within 2 to 3 h after death of the animal. In the laboratory, ovaries were rinsed 3 times in saline at 35℃ and attached tissues were removed by using a sterile scissors.

Follicular fluid was aspirated from individual visible follicles of 2 to 8 mm diameter by using an 18-gauge needle (TERUMO®, Beijing, China) attached to a 10-ml disposable plastic syringe (Steripack Disposable Syringe®, Opso Saline Ltd, Dhaka, Bangladesh). The retrieved follicular fluid was left for 5 minutes; the sediment was then transferred to a 60 mm Petri dish (Greiner bio-one, Frickenhausen, Germany) and diluted with TL-HEPES (Bioniche, Animal Health Inc., Pullman, WA, USA) and searched for COCs under a sterio-microscope (Leica Microsystems, MZ6, Wetzlar, Germany). The COCs were washed 3 times in fresh TL-HEPES and once in maturation medium before putting those in the maturation drops. Oocytes having multilayered compact cumulus investment and homogenous ooplasm were selected for *in vitro*-maturation.

The basic medium used for oocyte maturation was tissue culture medium-199 (TCM, Earle's salts with L-glutamine and sodium bicarbonate, Gibco®, Invitrogen Corporation, NY, USA). On the day of maturation, TCM was supplemented with sodium pyruvate (0.25 mM), fetal bovine serum (FBS, 10%, v/v, Gibco®, USA), bovine FSH (0.05 μg/ml, Sioux Biochemical, Sioux center, Iowa, USA), LH (5 μg/ml, Sioux Biochemical, USA), oestradiol (1 μg/ml) and gentamycin (50 μg/ml). Four 50 μl drops of maturation medium were prepared in a 35 mm petri dish (Greiner bio-one, Frickenhausen, Germany) and covered with mineral oil. Ten to 12 washed oocytes were placed in each drop and cultured in the incubator for 24 h in 5% CO_2 in humidified air at 39℃.

2. Evaluation of Oocytes for Maturation

At 24 h maturation, oocytes were either fixed or fertilised. To evaluate the oocyte maturation, the expanded oocytes were removed from maturation drops, vortexed for 3 minutes in 500 μl TL-HEPES to remove cumulus cells. The denuded oocytes were recovered under a stereomicroscope, mounted between a cover slip and a slide, fixed in acetic acid: ethanol (1:3) for 24 h. After fixation, oocytes were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 10 minutes and distained in acetic acid: glycerol: distilled water (1:1:3) to evaluate nuclear stages of oocytes under a microscope (OLYMPUS®, BX51, Tokyo, Japan) equipped with differential interference contrast (DIC) at 400 × (Ali and Sirard, 2002). Oocytes showing metaphase Ⅱ (MⅡ) stage and a first polar body (PB) were considered as matured (Shamsuddin *et al.*, 1993a), while oocytes with germinal vesicle (GV) / germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI) or telophase I (TI) stage were considered as not matured. Oocytes showing an abnormal chromatin configuration or no chromatin at all after staining were considered as being degenerated and were discarded.

3. Sperm Preparation and *In Vitro* Fertilisation

Frozen semen from 14 bulls (7 zebus and 7 crossbreds) from Central AI Laboratory, Savar, Dhaka, Bangladesh was used in this study. Two straws (0.25-ml straws, 25 $\times 10^6$ sperm/ straw) from each bull were thawed at 37℃ for 12 seconds. The thawed semen (500 μl) was layered on a discontinuous gradient column of Percoll in a 15 ml tube (Bottom layer: 2 ml, 90%, v/v; Top layer: 2 ml, 45%, v/v). The tube was spanned down at 900 g for 15 minutes in a centrifuge machine (Centra-CL2®, International Equipment Company, Massachusetts, USA). From the bottom of the test tube, 100 μl of the sperm pellet was aspirated, transferred to an eppendorf tube. Sperm concentration in the pellet was determined by using a hemocytometer (Bane, 1952) and adjusted to 1.0×10^6/ml with IVF-TL (Embryomax®, Marshall Street, Philisburg, NJ), which was a Tyrode's lactate solution, supplemented with fatty acid free BSA (6 mg/ml) and sodium pyruvate (0.25 mM). The motility of separated spermatozoa used for insemination was evaluated within 5 minutes of semen separation. For motility evaluation, 10 μl of semen was placed on to a pre-warmed slide at 37℃, covered with a warmed cover glass and evaluated under a microscope equipped with DIC optics at 200×. Motility was assessed sub-

jectively by 2 independent evaluators as percentages of spermatozoa moving progressively straight forward.

IVF-TL was used for sperm-oocyte co-incubation. Forty-four microlitre drops of IVF-TL were prepared in 35 mm petridish, covered with mineral oil and equilibrated in the incubator for 2 h. After 24 h maturation, the expanded COCs were removed from the IVM drops, washed 3 times in TL-HEPES, and once in IVF-TL. Ten COCs were transferred to individual drops of IVF-TL. Two-μl heparin (5 μg/ml), 2 μl PHE (D-penicillamine, 20 μM; hypotaurine, 10 μM; and epinephrine, 1 μM) and 2 μl sperm suspension were added in each fertilisation drop. Spermatozoa and COCs were co-incubated for 18 h in 5% CO_2 in humidified air at 39℃.

4. Evaluation of Oocytes for Fertilisation

After 18 h of sperm-oocyte co-incubation, the oocytes were vortexed for 3 minutes in 500 μl of TL-HEPES to remove cumulus cells and excess spermatozoa. The denuded presumptive zygotes were examined for pronuclei, as described for the evaluation of oocyte maturation. The presence of two pronuclei (2PN) within cytoplasm of an oocyte was considered as normal fertilisation (Shamsuddin *et al.*, 1993b; Tanghe *et al.*, 2002; Alomar *et al.*, 2008), while more than two pronuclei (> 2PN) was considered as polyspermia (Shamsuddin *et al.*, 1993b; Tanghe *et al.*, 2002). Oocytes with only one pronucleus (1PN) was considered as either an asynchronous/delayed pronucleus formation (Xu and Greeve, 1988) or parthenogenetic activation of oocytes (Staessen *et al.*, 1993) or because one pronucleus was obscured by lipid cytoplasmic droplets (Kubisch *et al.*, 1995).

5. Statistical Analysis

Statistical analysis was carried out by using statistical packages for social sciences (SPSS), version 10.0 (1999). Paired *t*-test was performed to detect differences between post-thaw sperm motility and motility after Percoll seperation of spermatozoa and *t*-test was done to identify differences in different fertilisation parameters between two groups of bulls. One-way analysis of variance (ANOVA) was done to find out the effects of individual bull on *in vitro* fertilisation.

RESULTS

The mean ± SD number of oocytes retrieved from each ovary was 5.4 ± 0.7. The detailed result of IVM is presented in Table 1. The percentage of matured oocytes was 73.0. An *in vitro* matured oocyte with a polar body and metaphase Ⅱ (MⅡ) stage chromosomes is shown in Fig. 1.

The post-thaw sperm motility was 44.8 ± 2.8 and 45.2 ± 3.1%, while the sperm motility increased by 76.7 ± 1.2 and 78.4 ± 3.1% after Percoll separation for zebu and crossbred bulls, respectively. The difference in motility of spermatozoa before and after Percoll seperation was significant (p<0.001). However, the difference between zebu and crossbred bulls with regard to sperm motility was not significant.

The results of IVF sired by zebu and crossbred bulls are shown in Fig. 2. The differences in different fertilisation parameters were not significant between two bull-groups (p>0.05).

The IVF result of individual bull is shown in Table 2. The mean ± SD percentages of oocytes penetrated by one or more spermatozoa ranged between 39.5 ± 8.7 and 92.9 ± 6.5; the proportion of normally fertilised oocytes (Fig. 3) varied from 31.3 ± 3.5 to 85.8 ± 8.3; percentages of polyspermia (Fig. 4) ranged from 5.5 ± 4.8 to 12.6 ± 6.8 and the number of oocytes showing only one pronucleus (Fig. 5) varied from 0.0 to 11.3 ± 5.1 % for all zebu bulls. The fertlisation parameters other than the

Table 1. Nuclear stages of zebu cattle oocytes at 24 h of IVM culture

No. of oocytes	No. of oocytes at different nuclear stages of			IVM rate
	GV/ GVBD	MI/AI /TI	MⅡ	
162	14	25	118	73.0 %

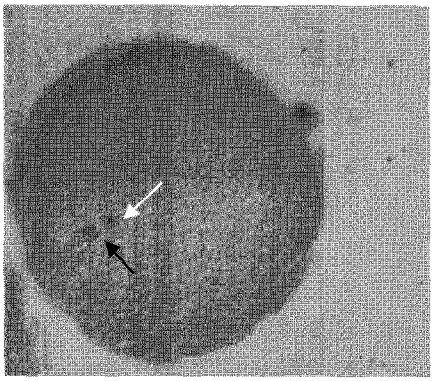

Fig. 1. An *in vitro* matured oocyte with a polar body (white arrow) and metaphase II stage chromosomes (black arrow) (1% aceto-orcein stain, DIC microscopy, 400×).

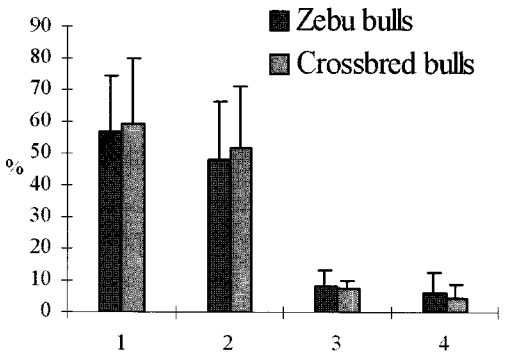

Fig. 2. Mean ± SD percentages of oocytes penetrated by one or more spermatozoa, normally fertilised oocytes, polyspermic oocytes and oocytes with one pronucleus at 18 h after IVF with semen from two groups of bulls. Number of bulls in each group = 7; the experiment was repeated 3 times with semen from each bull.

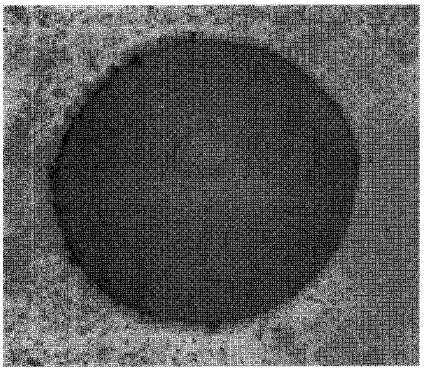

Fig. 3. Normally fertilised oocyte (1% aceto-orcein stain, DIC microscopy, 400 ×).

polyspermia varied between individual bulls ($p<0.05$, Table 2).

For crossbred bulls, percentages of sperm penetrated oocytes, normal fertilisation, polyspermia and oocytes with one pronucleus varied from 41.8 ± 3.8 to 87.7 ± 4.2, 34.9 ± 3.4 to 75.6 ± 3.1, 5.4 ± 4.0 to 19.0 ± 9.2, and 2.5 ± 0.3 to 12.8 ± 6.8, respectively. The fertilisation parameters differed between individual

Table 2. Effects of individual bull on normal and abnormal fertilisation at 18 h after IVF

Types of bulls	Bulls' ID	No of oocytes	Fertilisation parameters			
			Oocytes penetrated by one or more spermatozoa (%)	Oocytes fertilised normally (%)	Polyspermic oocytes (%)	Oocytes with one pronucleus (%)
Zebu	430	53	51.5 ± 8.8^{ab}	39.0 ± 5.0^{ab}	12.6 ± 6.8	11.3 ± 5.1^{b}
	536	51	47.9 ± 6.7^{a}	42.4 ± 4.7^{b}	5.5 ± 4.8	11.2 ± 8.4^{b}
	499	55	39.5 ± 8.7^{a}	31.3 ± 3.5^{a}	8.1 ± 5.8	10.8 ± 4.3^{ab}
	424	57	92.9 ± 6.5^{c}	85.8 ± 8.3^{d}	7.1 ± 2.8	0.0 ± 0.0^{a}
	541	56	65.4 ± 5.2^{b}	57.0 ± 1.9^{c}	8.5 ± 3.7	3.4 ± 2.4^{ab}
	313	58	48.4 ± 1.9^{a}	40.0 ± 2.1^{b}	6.9 ± 1.2	5.2 ± 2.1^{ab}
	419	60	50.3 ± 2.0^{a}	42.3 ± 0.3^{b}	6.3 ± 3.3	3.0 ± 1.6^{ab}
Crossbred	5156	58	41.8 ± 3.8^{a}	34.9 ± 3.4^{a}	6.9 ± 1.1^{a}	9.9 ± 1.6^{ab}
	9362	61	73.6 ± 5.6^{c}	54.6 ± 5.0^{c}	19.0 ± 9.2^{b}	6.8 ± 4.8^{ab}
	9386	54	57.3 ± 5.2^{b}	51.9 ±1.7^{bc}	5.4 ± 4.0^{a}	12.8 ± 6.8^{b}
	8269	58	58.2 ± 4.4^{b}	46.2 ± 4.5^{b}	12.0 ± 1.3^{ab}	8.7 ± 2.7^{ab}
	D214	53	87.7 ± 4.2^{d}	75.6 ± 3.1^{d}	12.1 ± 3.3^{ab}	7.8 ± 2.5^{ab}
	4813	65	47.5 ± 3.0^{a}	38.8 ± 3.5^{a}	7.4 ± 1.0^{a}	5.4 ± 2.0^{ab}
	4866	63	49.3 ± 4.2^{ab}	42.9 ± 2.4^{ab}	5.6 ± 2.7^{a}	2.5 ± 0.3^{a}

Values are the Mean ± SD.

No. of replicates for each bull = 3.

$^{a\sim c}$ Values having different superscripts within the same column differ significantly ($p<0.05$).

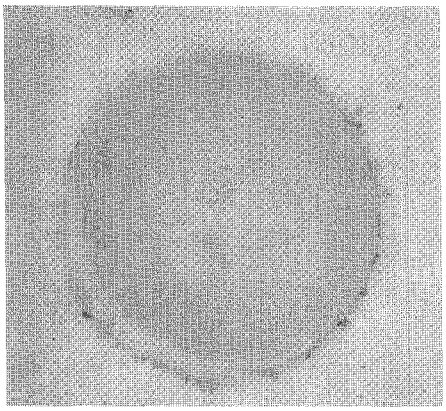

Fig. 4. Polyspermic oocyte (1% aceto-orcein stain, DIC microscopy, 400 ×).

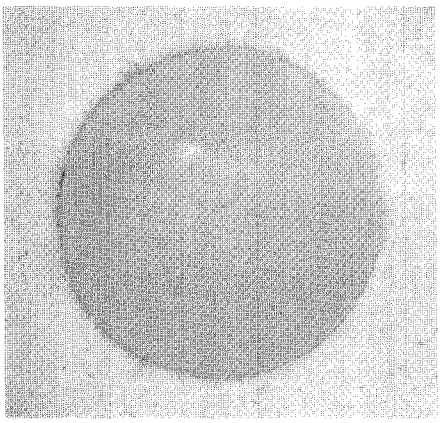

Fig. 5. An oocyte with one pronucleus (1% aceto-orcein stain, DIC microscopy, 400 ×).

bulls ($p<0.05$, Table 2).

DISCUSSION

Co-incubation of *in vitro* matured oocytes and spermatozoa in the laboratory often results in abnormal fertilisation, which partly explains the low percentages of embryo development and subsequent live birth of offspring when resulted embryos are transferred into foster mothers. Two experiments have already been conducted for the establishment of the techniques of *in vitro* maturation, fertilisation and culture of zebu cattle oocytes in Bangladesh (Das, 2005; Islam *et al.*, 2007). This study examined the nuclear stages of *in vitro* matured oocytes in Bangladeshi zebu cattle and pronucleus formation ability of such oocytes after IVF.

In the present study, the percentages of sperm penetrated oocytes as well as normal fertilisation rate varied depending on individual bulls. Some bulls achieved quite high percentages of fertilisation and few bulls showed low fertilisation rate *in vitro*. This result is similar to finding of other researchers and they reported that spermatozoa from different bulls differ in their ability to fertilise oocytes *in vitro* (Marquant-Le Guienne *et al.*, 1993, Shamsuddin and Larsson, 1993; Ward *et al.*, 2001). Moreover, spermatozoa from different semen lots and even straws within the semen lot from a single bull differ when used in *in vitro* fertilisation (Otoi *et al.*, 1993).

In this study, a large proportion of oocytes exhibited polyspermic fertilisation. The sperm concentration may have a significant influence on polyspermic fertilisation. Hence a negotiation must be found having a sufficient number of sperm to ensure acceptable fertilisation rates while ensuring a minimal incidence of polyspermy. In bovine IVF, commonly used sperm concentration ranged between 0.5×10^6 and 5×10^6 spermatozoa/ ml (Gordon, 1994). In the present experiment, a concentration of 1.0×10^6 spermatozoa/ml was used for IVF. Van der Ven *et al.* (1985) reported that the polyspermy rate was affected by the number of spermatozoa used for *in vitro* fertilisation. Insemination with 0.5 to 0.8, 1.0, or 1.5 to 2.0×10^6 spermatozoa / oocyte resulted in a polyspermy rate of 6%, 20%, and 32%, respectively. Some authors have reported an increase in the rate of polyspermy at high concentrations and high sperm: oocyte ratios (Chian *et al.*, 1992; Sumantri *et al.*, 1997). However, the effect of polyspermic fertilisation on subsequent embryo development is still inconclusive (Lightfoot *et al.*, 2006).

Van der Ven *et al.* (1985) stated that the frequency of polyspermy was related to the maturity of the oocyte, determined according to morphologic criteria. Immature oocytes showed a higher percentage of polyspermic fertilisation (32%) compared to that of mature oocytes (6%). An incomplete or abnormal cytoplasmic maturation could explain the high rates of polyspermic fertilisation in this study. Improving oocyte maturation, reducing the number of spermatozoa per oocyte in an IVF drop and the exact timing of insemination according to the maturity of the oocyte might reduce the occurrence of polyspermic fertilisation.

Number of oocytes showing only one pronucleus at 18 h post-IVF varied from 0.0 to 12.8 ± 6.8 %, depending on individual bulls used in this study ($p<0.05$). The presumptive zygotes visualized with a single pronucleus suggests that formation of second pronucleus might be delayed, possibly because of late maturation, or subsequent delayed fertilisation, or both. In addi-

tion, parthenogenetic activation of oocytes may lead to development of one pronucleus or because one pronucleus was obscured by cytoplasmic lipid droplets. Previous two studies reported that in 25% of single pronucleated oocytes a second pronucleus was observed 4~6 h later, suggesting asynchronous or delayed pronuclear formation (Staessen *et al.*, 1993; Chian *et al.*, 1995). Parthenogenetic activation or asynchronous pronuclei development may both be mechanisms leading to the morphological observation of a single pronucleus (Staessen *et al.*, 1993). However, two authors reported no significant difference in the cleavage rates between zygotes with 1PN or 2PN at 16 to 18 h after insemination (Staessen *et al.*, 1993; Chian *et al.*, 1995). Moreover, Kubisch *et al.*, (1995) reported that 67% cleavage rates was observed in the presumptive zygotes in which pronuclei were not visualized at 19 h after *in vitro* insemination. Others reported that only 19% presumptive zygotes with no visible pronucleus were cleaved (Chian *et al.*, 1995). Whether such differences are due to evaluator-related variations, remained to be determined. In this study, no attempt was taken to allow zygotes for embryonic development. However, it appears that the presence of only one pronucleus is not an indication of fertilisation failure.

Here the discontinuous Percoll gradients centrifugation method was used for sperm separation. The difference in motility of spermatozoa before and after Percoll seperation was significant ($p<0.001$). This indicates that, Percoll density gradient centrifugation could be an effective method of sperm preparation for optimization of highly motile spermatozoa. However, there is a report of lower fertilisation rate by Percoll separated spermatozoa than that by swim-up separated spermatozoa when same sperm concentrations were used (Avery *et al.*, 1995). In contrast, in swim-up method, centrifugation twice and rinsing in Sperm-TALP could reduce the selected spermatozoa by up to 20%. (Shamsuddin *et al.*, 1993b).

The first step towards *in vitro* fertilisation is oocytes maturation. We used a heterogeneous population of oocytes derived from slaughterhouse ovaries regardless of follicular dynamics and age of the oocyte donors. However, a good percentage of oocytes matured *in vitro* in this study. The proportion of oocytes matured in this study seems to be higher than that of a previous study in this laboratory (Das, 2005). The percentage of matured oocytes in this study is also comparable with that of other research findings (Shamsuddin *et al.*, 1993a; Fortune, 1994; Camargo *et al.*, 2005; Islam *et al.*, 2007). *In vitro* maturation of oocytes is influenced by the supplementation of gonadotrophins in TCM 199 medium where LH alone failed to improve the oocyte maturation; however, the addition of FSH and oestradiol increased the proportion of matured oocytes (Totey *et al.*, 1993). In this study, we used both FSH and LH as gonadotrophin stock.

In present study, mean number of oocytes retrieved from an ovary was 5.4 ± 0.7. The proportion of oocytes retrieved per ovary in this study seems to be higher than that of previous studies in this laboratory (Das, 2005; Islam *et al.*, 2007). Moreover, the average number of oocyte retrieved from an ovary in this study is also comparable with that of another study with zebu cattle (Dode *et al.*, 2001). Such differences might be due to individual variation, seasons and quality of the ovaries collected.

In conclusion, a protocol for IVF in Bangladeshi zebu cattle is established; however, a large proportion of oocytes exhibited abnormal fertilisation, including polyspermia and oocytes with one pronucleus. The higher percentages of abnormal fertilisation may be attributed to abnormal maturation and indicated the protocol needs for improving IVM and defining sperm number with regards to consistent IVF results in reducing abnormal fertilisation. A future study may elucidate the capacity of such IVM-IVF oocytes to obtain blastocysts to transfer to surrogate mother.

REFERENCES

Alam MGS. 2001. Selection and improvement of cows. In: Cattle Fertility and General Disease Control. 1st ed, Published by Setara Alam, E-16/4, Residence area, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Ali A and Sirard MA. 2002. Effect of the absence or presence of various protein supplements on further development of bovine oocytes during *in vitro* maturation. Biol. Reprod. 66:901-905.

Alomar M, Tasiaux H, Remacle S, George F, Paul D and Donnay I. 2008. Kinetics of fertilisation and development, and sex ratio of bovine embryos produced using the semen of different bulls. Theriogenology 107:48-61.

Avery B, Brandenhoff HR and Greve T. 1995. Development of *in vitro* matured and fertilised bovine embryos cultured from days 1-5 post insemination in either Menezo-B2 medium or in HECM-6 medium. Theriogenology 44:935-945.

Bane A. 1952. A study on the technique of hemocytometric determination of sperm motility and sperm concentration in

bull semen. The Cornell. Vet. 42:518-531.

Camargo LS, Viana JHM, Sa WF, Ferreira AM and Vale VR. 2005. Developmental competence of oocytes from prepubertal *Bos indicus* crossbred cattle. Anim. Reprod. Sci. 85:53-59.

Chian RC, Niwa K and Nakahara H. 1992. Effect of sperm penetration *in vitro* on completion of first meiosis by bovine oocytes arrested at various stages in culture. J. Reprod. Fertil. 96:73-78.

Chian RC, Okuda K and Niwa K. 1995. Influence of cumulus cells on *in vitro* fertilisation of bovine oocytes. Anim. Reprod. Sci. 38:37-48.

Das ZC. 2005. *In vitro* fertilisation of bovine oocytes using zebu and crossbred bull semen. MS in Theriogenology Thesis, Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. pp. 1-53.

Dode MAN, Rodovalho NC, Ueno VG and Alves RGO. 2001. Oocyte recovering from *Bos indicus* female. Arch. Zootec. 50:415-418

Fortune JE. 1994. Ovarian follicular growth and development in mammals. Biol. Reprod. 50:225-232.

Gordon I. 1994. Laboratory production of cattle embryos. In: Biotechnology in Agriculture, 11. I. Gordon (Editor). CAB International, Wallingford, p. 640.

Islam MF, Bhuiyan MMU, Banu TA, Bhattacharjee J and Shamsuddin M. 2007. *In vitro* maturation and fertilisation of oocytes in zebu cattle. Bangladesh Vet. J. 41:58-76.

Kubisch HM, Larson MA, Funahashi H, Day BN and Roberts RM. 1995. Pronuclear visibility, development and transgenic expression in IVM/IVF derived porcine embryos. Theriogenology 44:391-401.

Lightfoot DA, Kouznetsova A, Mahdy E, Wilbertz J and Hoog C. 2006. The fate of mosaic aneuploid embryos during mouse development. Dev. Biol. 289:384-394.

Marquant-Le Guienne B, Humblot P, Thibier M and Thibault C. 1993. Evaluation of bull semen fertility by homologous *in vitro* fertilisation test. Reprod. Nutr. Dev. 30:259-266.

Otoi T, Tachikawa S, Kondo S and Suzuki T. 1993. Effects of different lots of semen from the same bull on *in vitro* development bovine oocytes fertilised *in vitro*. Theriogenology 39:713-718.

Parrish JJ, Susko-Parrish JL, Winer MA and First NL. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Shamsuddin M and Larsson B. 1993. *In vitro* development of bovine embryos after fertilisation using semen from different donors. Reprod. Dom. Anim. 28:77-84.

Shamsuddin M, Larson B and Rodriguez-Martinez H. 1993a. Maturation related changes in bovine oocytes under different culture conditions. Anim. Reprod. Sci. 31:49-60.

Shamsuddin M, Rodriguez-Martinez H and Larsson B. 1993b. Fertilising capacity of bovine spermatozoa selected after swim-up in hyaluronic acid containing medium. Reprod. Fertil. Dev. 5:307-315.

Staessen C, Janssenswillen P, Devroey P and Van Steirteghen AC. 1993. Cytogenetic and morphological observations of single pronucleated human oocytes after *in vitro* fertilisation. Hum. Reprod. 8:221-223.

Sumantri C, Boediono A, Ooe M, Murakami M, Saha S and Suzuki T. 1997. The effect of sperm-oocyte incubation time on *in vitro* development using sperm from a tetraparental chimeric bull. Anim. Reprod. Sci. 48:187-195.

Syrstad O. 1996. Dairy cattle crossbreeding in the tropics: Choice of crossbreeding strategy, Trop. Anim. Health. Prod. 28: 223-229.

Tanghe S, Van Soom A, Stercks V, Maes D and Kruif A de. 2002. Assessment of different sperm quality parameters to predict *in vitro* fertility of bulls. Reprod. Dom. Anim. 37: 127-132.

Totey SM, Pawshe CH and Singh GP. 1993. *In vitro* maturation and fertilisation of buffalo oocytes (*Bubalus bubalis*): Effects of media, hormones and sera. Theriogenology 39: 11531171.

Van der Ven HH, Al-Hasani S, Diedrich K, Hamerich U, Lehmann F and Krebs D. 1985. Polyspermy in *in vitro* fertilisation of human oocytes: frequency and possible causes. Ann. New York Acad. Sci. 442:88-95.

Ward FA, Rizos D, Corridan D, Quinn K, Boland MP and Lonergan P. 2001. Paternal influence on time of the first embryonic cleavage post insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. Mol. Hum. Reprod. 60:47-55.

Xu KP and Greve T. 1988. A detailed analysis of early events during *in vitro* fertilisation of bovine follicular oocytes. J. Reprod. Fertil. 82:127-134.

(접수일: 2009. 3. 28 / 채택일: 2009. 5. 7)