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Normal and Abnormal Fertilisation of Zebu Cattle Oocytes *in-vitro*

AK Talukder, M Shamsuddin, MB Rahman, FY Bari, JJ Parish

Department of Surgery and Obstetrics, Faculty of Veterinary Science,

Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

ABSTRACT

Successful embryo production relies on the normal maturation and fertilisation of oocytes *in vitro*. This study reports *in vitro* fertilisation (IVF) of *in vitro* matured (IVM) zebu cattle oocytes with emphasis on abnormalities. Immature cumulus oocyte complexes (COCs) from zebu cows' ovaries at slaughter were matured *in vitro* for 24h, fertilised with Percoll-separated, heparin-treated spermatozoa (1.0 10^6/mL) at 39 ºC with 5% CO_2 in humidified air. After 24 h culture, COCs were examined for expansion and oocytes were denuded, fixed with acetic acid-alcohol, stained with aceto-orcein and examined under the microscope (×100-400) for metaphase II to confirm maturation. After 18 h of sperm-COC co-incubation, the presumptive zygotes were fixed, stained and examined accordingly for pronuclei.

The mean ± SD number of oocytes retrieved from an ovary was 5.4± 0.7. The percentage of matured oocytes was 73.0 ± 13.4. The difference in motility of spermatozoa before and after Percoll seperation was significant ($p< 0.0001$) for all zebu and crossbred bulls.The percentages of sperm-penetrated oocytes, normally fertilised oocytes, polyspermic oocytes and oocytes with one pronucleus sired by semen of zebu bulls (n = 5) and crossbred bulls (n = 5) were 59.4 ± 20.9, 51.1 ± 21.5, 8.4 ± 2.6, 7.3 ± 5.3 and 63.7 ± 15.7, 52.6 ± 13.3, 11.1 ± 4.8, 9.2 ± 2.1, respectively. The differences in fertilisation parameters were not significant between two bull-groups ($P> 0.05$). Pooling data from all 10 bulls, the mean ± SD percentages of sperm-penetrated oocytes ranged between 39.5 ± 8.7 and 92.9 ± 6.5; the proportion of normally fertilised oocytes ranged from 31.3 ± 3.5 to 85.8 ± 8.3 and percentages of polyspermia varied from 5.4 ± 4.0 to 19.0 ± 9.2. The number of oocytes with only one pronucleus (a possible indication of parthenogenetic development), ranged from 0.0 to 12.8 ± 6.8%. The fertilisation parameters differed among individual bulls ($P<0.05$). A protocol for *in vitro* fertilisation of *in vitro* matured zebu cattle oocytes has been developed. A future study may elucidate the capacity of such IVM-IVF oocytes to develop to full term.

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*Corresponding author: Telephone: 880 17 11 156308; Fax: + 880 91 55810;
e-mail: m.shamsuddin@gmail.com

Introduction

In vitro fertilisation (IVF), an assisted reproductive technology in which eggs are fertilised outside a female's womb, has been used extensively in embryo research in many countries since the late eighties but IVF protocols for zebu cattle embryos production is still under research. Milk production in tropically adapted non-descript zebu (*Bos indicus*) cows of Bangladesh is low compared with that of exotic (*Bos taurus*) one in their natural habitant region of the world. However, the *Bos taurus* cattle have been continuously failing to show such productivity in many of the tropical countries (Syrstad, 1996). The first crossbred generation (F_1 cows) derived from zebu females mated with exotic bulls, on the other hand, have shown a better adaptability and produced more milk at tropics (Syrstad, 1996; Alam, 2001). In conventional artificial insemination (AI) technique, breeding an F1 cow to produce an F1 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 F1 embryos.

The first calf produced from *in vitro* fertilisation of an oocyte matured in vivo was a male born in 1981 (Brackett *et al.*, 1982). Since then, considerable effort has been devoted to developing and improving *in vitro* maturation and *in vitro* fertilisation of oocytes as well as *in vitro* culture of zygotes for the last two erra. The reason for the considerable interest in this technology is the ability to produce at low cost synchronized embryos at a specific stage of development [Ref]. However, now a days, the IVF technology in domesticated animals particularly in cattle, has been used for the production of embryos in regular breeding programme (dairy and beef purpose), to overcome reproductive disorders in cows, predicting bull fertility, in cloning technology and in stem cell research. But there are only few reports on IVF in zebu or crossbred cattle in comparison to that in *Bos taurus* cattle.

An attempt was taken to establish a protocol of *in vitro* maturation, fertilisation and culture of zebu cattle oocytes for the first time in Bangladesh 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 had been made, 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 zebu cattle oocytes and normal and abnormal fertilisation of such oocytes *in vitro*

Materials and Methods

The present study was conducted at the Field Fertility Clinic Laboratory, Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh during the period from January 2007 to April 2008.

Chemicals and media used

Unless otherwise mentioned, all the chemicals, reagents, media, 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*., 1988b). The final media for maturation and fertilisation were filtered (0.22 µm poor size, Durapure® membrane filter, Ireland) and routinely equilibrated at 39° C 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.

***In vitro* maturation of oocytes**

Collection of ovaries

Ovaries of cattle were collected at slaughter and transported to the laboratory in a thermo flask containing warm saline (35 C, 0.9% sodium chloride solution, w/v), supplemented with penicillin-streptomycin (125 g / 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°C and attached tissues were removed by using a sterile scissors.

Oocytes retrieval

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 retrived 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 cumulus-oocyte complexes (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.

Culture of oocytes for 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®, Invitrogen Corporation, NY, USA), bovine FSH (0.05 µg/ml, Sioux Biochemical, Sioux center, Iowa, USA), LH (5 µg/ml, Sioux Biochemical, Sioux center, Iowa, 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 39º C.

Evaluation of oocytes for maturation

At 24 h maturation, the expanded oocytes were removed from maturation drops, vortexed for 3 minutes in 500 l TL-HEPES to remove cumulus cells. The denuded oocytes were recovered under a stereomicroscope, mounted between a coverslip and a slide, fixed in acetic acid: ethanol (1:3) for 24 h and stained with1% (w/v) aceto-orcein in 40% acetic acid for 5 minutes to observe metaphase II chromosome stage and polarbody under a

microscope (OLYMPUS®, BX51, Tokyo, Japan) equipped with differential interference contrast (DIC) at 400×. Oocytes showing metaphase II stage and a first polarbody (PB) were considered as matured [ref], while oocytes with germinal vesicle (GV)/germinal vesicle breakdown (GVBD), metaphase I stage or picnotic chromatin were considered as not matured.

In vitro fertilisation

Semen

Frozen semen from10 bulls from Central AI Laboratory, Savar, Dhaka, Bangladesh (5 zebus and 5 crossbreds (Friesian sires and Zebu dams)) was used in this study.

Sperm preparation

Two straws (0.25-ml straws, 25 $\times 10^6$ sperm/ straw) from each bull were thawed at 37°C for 30 seconds. The thawed semen (500 l) 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 900g 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 $\times 10^6$/ mL with IVF-TL, which was a Tyrode's lactate solution, IVF-TL (Embryomax , Marshall Street, Philisburg, NJ), 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 37º C, covered with a warmed cover glass and evaluated under a microscope equipped with differential interference contrast optics at 200 ×. Motility was assessed subjectively by 2 independent evaluators as percentages of spermatozoa moving progressively straight forward.

In vitro insemination

IVF-TL was used for sperm-oocyte co-incubation. Forty-four microlitre drops of IVF-TL were prepared in 35 mm petri dish, 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, 20µM; 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 39º C.

Evaluation of oocytes for fertilisation

After18 h of sperm-oocyte co-incubation, the oocytes were vortexed for 3 minutes in 500 l of TL-HEPES to remove cumulus cells and excess spermatozoa. The denuded presumptive zygotes were recovered under a stereomicroscope, mounted between a coverslip and a slide and fixed in acetic acid: ethanol (1:3) for 24 h. Presumptive zygotes were then stained with 1% (w/v) aceto-orcein for 5 minutes to visualize pronucleus formation under a microscope equipped with differential interference contrast at 400× (Ali and Sirard, 2002). The presence of two pronuclei (2PN) within cytoplasm of an oocyte was considered as normal fertilisation (Shamsuddin *et al*., 1993c; Tanghe *et al*., 2002;

Alomar *et al*., 2008), while more than two pronuclei (>2PN) within cytoplasm of an oocyte was considered as polyspermia (Shamsuddin *et al*., 1993c; 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).

Statistical analysis

The data were recorded in Microsoft Excel work sheet and descriptive statistics were computed. Paired T test was performed to detect differences between post thaw sperm motility and motility after Percoll seperation of spermatozoa and to detect differences in different fertilisation parameters between two groups of bulls. One way analysis of variance (ANOVA) was carried out by using SPSS Version 16.0 (2007) to identify effect of bulls on different parameters of fertilisation.

Results

The mean ± SD number of oocytes retrieved from an ovary was 5.4± 0.7. The detailed results of IVM is presented in Table 1.The percentage of matured oocytes was 73.0 ± 13.4. The maturation of the oocytes was confirmed by observing metaphase II (MII) stage and first polar body in the cytoplasm of oocytes (Figure 1).

The post-thaw sperm motility and sperm motility after Percoll seperation, of individual bulls is summarized in the Table 2. The post-thaw sperm motility was 45.0 ± 3.0 and 45.7 ± 3.3%, while the sperm motility increased by 76.8 ± 1.8 and 78.9 ± 3.0 % after Percoll separation for zebu and crossbred bulls, respectively. The difference in motility of spermatozoa before and after Percoll seperation was significant ($p < 0.0001$) for all zebu and crossbred bulls.

The percentages (mean ± SD) of sperm-penetrated oocytes sired by semen of zebu bulls (n = 5) and crossbred bulls (n = 5) were 59.4 ± 20.9 and 63.7 ± 15.7, respectively. The percentages of normally fertilised oocytes were 51.1 ± 21.5 and 52.6 ± 13.3; the proportion of polyspermic oocytes were 8.4 ± 2.6 and 11.1 ± 4.8 and percentages of oocytes showing one pronucleus were 7.3 ± 5.3 and 9.2 ± 2.1 for zebu and crossbred bulls, respectively. The differences in different fertilisation parameters were not significant between two bull-groups (Table 3).

The details of IVF results are shown in Table 4. Pooling data from all 10 bulls, 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 (oocytes that possessed two pronuclei, Figure 3) varied from 31.3 ± 3.5 to 85.8 ± 8.3 and percentages of polyspermia (oocytes with more than two pronuclei, Figure 4) ranged from 5.4 ± 4.0 to 19.0 ± 9.2. The number of oocytes showing only one pronucleus (a possible indication of parthenogenetic development) varied from 0.0 to 12.8 ± 6.8 % (Figure 5). The fertlisation parameters differed between individual bulls and different replicates of experiments.

Table 1. *In vitro* maturation stages of zebu cattle oocytes after incubation in TCM 199 medium in the incubator for 24 h in 5% CO_2 in humidified air at 39º C. Number of oocytes examined = 118; number of replicates = 9.

	Developmental stages		
	GV /GVBD	Metaphase I	Metaphase II
Percentage (mean ± SD)	8.6 ± 6.4	15.3 ±9.2	73.0 ± 13.4

Table 2. Mean ± SD post-thaw sperm motility and sperm motility after Percoll seperation with regards to zebu and crossbred bulls used in IVF. Number of replicates for each bull = 3.

Types of bulls	Bulls' ID	Sperm motility (%)	
		Post-thawing	After Percoll seperation
Zebu	424	50.0 ^{a} ± 5.0	78.3 ^{b} ± 2.9
	541	43.3 ^{a} ± 2.9	78.3 ^{b} ± 2.9
	430	43.3 ^{a} ± 2.9	75.7 ^{b} ± 2.9
	536	46.7 ^{a} ± 5.8	76.7 ^{b} ± 2.9
	499	41.7 ^{a} ± 2.9	75.0 ^{b} ± 5.0
	Total	45.0 ^{a} ± 3.0	76.8 ^{b} ± 1.8
Crossbred	D214	50.0 ^{a} ± 5.0	83.3 ^{b} ± 2.9
	9386	43.3 ^{a} ± 5.8	75.0 ^{b} ± 5.0
	5156	43.3 ^{a} ± 5.8	78.3 ^{b} ± 2.9
	9362	48.3 ^{a} ± 2.3	78.3 ^{b} ± 2.3
	8269	43.3 ^{a} ± 2.9	80.0 ^{b} ± 5.0
	Total	45.7 ^{a} ± 3.3	78.9 ^{b} ± 3.0

Values with different superscripts (a,b) in the same row differ significantly (P< 0.05)

Table 3. Percentages (Mean ± SD) of bovine oocytes penetrated by one or more spermatozoa, fertilised normally (2 PN), fertilised by more than one spermatozoon (>2 PN) and with one pronucleus (1 PN) 18 h after in vitro fertilisation. Number of replication for each bull = 3.

Types of bulls	Bulls' ID	No of oocytes	Oocytes penetrated by one or more spermatozoa	Oocytes with 2PN	Oocytes with >2PN	Oocytes with1 PN
			Percentage (mean ± SD)			
Zebu	430	53	51.5 ^{ab} ± 8.8	39.0 ^{ab} ± 5.0	12.6 ± 6.8	11.3 ^{b} ± 5.1
	536	51	47.9 ^{a} ± 6.7	42.4 ^{b} ± 4.7	5.5 ± 4.8	11.2 ^{b} ± 8.4
	499	55	39.5 ^{a} ± 8.7	31.3 ^{a} ± 3.5	8.1 ± 5.8	10.8 ^{ab} ± 4.3
	424	57	92.9 ^{c} ± 6.5	85.8 ^{d} ± 8.3	7.1 ± 2.8	0.0 ^{a} ± 0.0
	541	56	65.4 ^{b} ± 5.2	57.0 ^{c} ± 1.9	8.5 ± 3.7	3.4 ^{ab} ± 2.4
Crossbred	5156	58	41.8 ^{a} ± 3.8	34.9 ^{a} ± 3.4	6.9 ^{a} ± 1.1	9.9 ± 1.6
	9362	61	73.6 ^{c} ± 5.6	54.6 ^{c} ± 5.0	19.0 ^{b} ± 9.2	6.8 ± 4.8
	9386	54	57.3 ^{b} ± 5.2	51.9 ^{bc} ± 1.7	5.4 ^{a} ± 4.0	12.8 ± 6.8
	8269	58	58.2 ^{b} ± 4.4	46.2 ^{b} ± 4.5	12.0 ^{ab} ± 1.3	8.7 ± 2.7
	D214	53	87.7 ^{d} ± 4.2	75.6 ^{d} ± 3.1	12.1^{ab} ± 3.3	7.8 ± 2.5

Values having different superscripts (a, b, c) are staistically significant ($P < 0.05$).

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 taken 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), but this is the first study with the purpose to examine developmental stages of *in vitro* matured zebu cattle oocytes and pronucleus formation ability of given bulls.

In the present study, the percentages of sperm penetrated oocytes varied depending on the bull used. Some bulls achieved quite high percentages of fertilisation and few bulls showed low fertilisation rate *in vitro*. However, a large proportion of oocytes exhibited abnormal fertilisation, including polyspermia and oocytes with only one pronucleus. Oocytes with only one pronucleus could be among others an indication of parthonogenetic development.

In vitro fertilisation is a multifactorial event that makes maintaining a consistent condition for all replicates difficult. This emphasizes the need for a stable condition to maintain for the IVF system. In addition, the variations could be due to the bull to bull variation. It is well known 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). Even spermatozoa from different semen lots and even straws within the semen lot from a single bull were shown to differ when used in *in vitro* fertilisation (Otoi *et al*., 1993).

The sperm penetration rate at 3h post-IVF was low, while it increased from 6 to 12h, but it remained the same for the following incubation time (12 and 18h; Dode *et al*., 2002). Furthermore, Iwata *et al*. (2008) reported that when the sperm-COC co-incubation was increased from 1 to 18h, the incidence of polyspermic fertilisation increased from 6.5% to 41.8% and the percentage of non-fertilised oocytes decreased from 36.8% to 8.5%. In contrast, Dode *et al*. (2002) stated that the sperm-oocyte co-incubation time did not influence the pronucleus formation or the polyspermy rate. However, Hyttel *et al*. (1988) appeared the first fertilised ovum at 8 h post-IVF and the first fully developed spherical pronucleus and at 20 h the first apposition of pronuclei was seen. On the other hand, it has been reported that the pernentages of zygotes showing two pronuclei had a maximum (70%) at 14h after fertilisation *in vitro* (Suraeva *et al*., 1997). The percentages of cleaved embryos gradually increased from 3 to 18h of sperm-oocyte co-incubation time, although no statistical differences were found between 12 and 18 h of incubation (Dode *et al*., 2002).

In bovine *in vitro* fertilisation, commonly used sperm concentration ranged between 0.5×10^6 and 5×10^6 spermatozoa/ml (Gordon, 1994). The sperm concentration can significantly influence the proportion of oocytes undergoing cleavage and development to the blastocyst stage. Hence a negotiation must be found having a sufficient number of sperm to ensure acceptable fertilisation rates while ensuring a minimal incidence of polyspermy. Van der ven (1985) reported that the polyspermy rate was affected by the number of spermatozoa used for *in vitro* fertilisation. Insemination with 0.5-0.8, 1.0, or 1.5-2.0 10^6 spermatozoa / oocyte resulted in a polyspermy rate of 6%, 20%, and 32%, respectively. In the present experiment, a concentration of 1.0×10^6 spermatozoa/ ml was

used. On the contrary, Ward *et al.* (2002) reported that a sperm concentration of 0.5×10^6 was superior to a concentration of 1.0×10^6 spermatozoa/ ml. 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 remains to be clarified (Lightfoot *et al*., 2006)

Van der ven (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 18h post-IVF varied from 0.0 to 12.8 ± 6.8 %, depending on individual bull used; however, difference between two bulls groups was not significant. The presumtive 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 addition, parthenogenetic activation of oocytes may lead to development of one pronucleus or because one pronucleus was obscured by cytoplasmic lipid droplets. Moreover, 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). No significant difference was observed in the cleavage rates between zygotes with 1PN or 2PN at the first evaluation (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 19h 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 made to allow zygotes for embryonic development. However, it appears that the presence of only one pronucleus is not an indication of fertilisation failure.

The first step towards *in vitro* fertilisation is oocytes maturation. 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 groups (Rocha *et al.,* 1998; Fortune, 1994; Camargo *et al.,* 2005; Islam, 2006). *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 present study, number (mean SD) 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, 2006). 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). The number of oocytes per ovary may

be influenced by season, being lower during the dry season (Dode *et al.,* 2001). The number of oocytes retrived is limited by the total number of follicles available for aspiration in the ovaries. Fortune (1994) stated that an increase in FSH levels in blood results in the recruitment of a pool of follicles and initiation of a new follicular wave and thereby influence the number of oocytes retrieved. Season throughout factors such as, nutrition and temperature can influence the gonadotrophins concentrations, which can affect the population of follicles recruited (Zeitoun *et al.,* 1996). These can explain the lower number of oocytes recovered during the dry season, when animals suffer from feed restriction due to the limitation of feed availability.

A highly heterogeneous population of oocytes derived from slaughter house ovaries, regardless of follicular dynamics, was used for *in vitro* fertilisation in this experiment. The proportion of embryos obtained from such oocytes reported insufficient with regard to the developmental competence and in average 20% reached blastocyst stage (Leibfried-Rutledge, 1999). It has been documented that large follicles contain more oocytes capable of developing into blastocysts than do smaller one (Pavlok *et al.,* 1992; Lonergan *et al.,* 1994a; Blondin and Sirard, 1995). The greater developmental competence of oocytes aspirated from larger follicles, as compared with smaller follicles was described by Johnson *et al.* (2001). Oocytes from prepubertal cattle have a lesser developmental competence than pubertal counterpart, possibly due to deficiencies in cytoplasmic maturation resulting in impaired embryo development (Mermillod *et al.,* 1998; Salamone *et al.,* 2001). However, this may vary depending on the age of the donor (Armstrong, 2001). Oocytes from prepubertal *B. indicus* crossbred heifers that are 9 months of age or more have the similar developmental competence after *in vitro* maturation, fertilisation and co-culture when compared with oocytes from cows, while oocytes from 4- to 7-month-old *B. indicus* crossbred calves are less competent for development to the blastocyst stage *in vitro* (Camargo *et al.,* 2005).

Here the discontinuous percoll gradients method of sperm separation was used as described by Parrish *et al.* (1995). The average post-thaw sperm motility was ranged between 41.7 ± 2.9 and 50.0 ± 5.0 %, while the sperm motility increased by 75.0 ± 5.0 to 83.3 ± 2.9 % after Percoll separation. This indicates that, Percoll density gradient centrifugation could be an effective method of sperm preparation for optimization of highly motile spermatozoa. The benefits of this method are simple to perform, require less time, highly motile and clean fraction of sperm can be harvested. Since the concentrated sperm pellet is harvested, it is always easy to adjust the concentration and inseminate with equal number of spermatozoa. However, there are reports 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 Sp-TALP could reduce the selected spermatozoa by up to 20%, irrespective of bull (Shamsuddin *et al*., 1993c).

In this study the heparin concentration used was 5 µg/ mL. It has been shown that capacitation of spermatozoa of different bulls or even different ejaculation of the same bull varied with the heparin concentration (Leibfried-Rutledge *et al*., 1989). However, Shamsuddin and Larsson (1993) have shown that 0.5-5 µg/mL of heparin did not show any significant difference in fertilisation rate. Moreover, Camargo *et al.* (2005) have shown that 20 µg/mL of heparin in the IVF drops achieved optimum fertilisation and development rate.

Conclusions

The results of the present study lead to the following conclusions:

1. A consistent IVF protocol was established, while few bulls showed quite high percentages of fertilisation and some bulls revealed low fertilisation rate. However, a large proportion of oocytes exhibited abnormal fertilisation, including polyspermia and oocytes with one pronucleus.
2. The fertilisation parameters differed among the individual bulls; however, the differences in different fertilisation parameters were not significant between two bull-groups.
3. The higher percentages of abnormal fertilisation indicated the protocol needs to improve IVM and to define sperm number with regards to consistent IVF results in reducing abnormal fertilisation.
4. A future study may elucidate the capacity of such IVM-IVF oocytes to develop to full term.

Reference

Ali A. and Sirard M.A. (2002). Effect of the absence or presence of various protein supplements on further development of bovine oocytes during *in vitro* maturation. *Biology of Reproduction* 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. *Theriogenoogy* 107: 48-61.

Armstrong D.T. (2001). Effect of maternal age on oocyte developmental competence. *Theriogenology* 55: 1303-1322.

Avery B., Brandenhoff H.R. 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 Veterinarian* 42: 518-531.

Blondin P. and Sirard M.A. (1995). Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular Reproduction and Development* 41: 54-62.

Brackett R.B., Bousquet D., Boice M.L., Donawick W.J., Evans J.F. and Dressel M.A. (1982). Normal development following *in vitro* fertilisation in the cow. *Biology of Reproduction* 27: 147-158.

Camargo L.S., Viana J.H.M., Sa W.F., Ferreira A.M. and Vale V.R. (2005). Developmental competence of oocytes from prepubertal *Bos indicus* crossbred cattle. *Animal Reproduction Science* 85(1-2): 53-9.

Chian R.C., 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. *Reproduction and Fertility* 96: 73-78.

Chian R.C., Okuda K. and Niwa K. (1995). Influence of cumulus cells on *in vitro* fertilisation of bovine oocytes. *Animal Reproduction Science* 38: 37-48.

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

Dode M.A., Rodovalho N.C., Ueno V.G. and Fernandes C.E. (2002). The effect of sperm preparation and co-incubation time on *in vitro* fertilisation of *Bos indicus* oocytes. *Animal Reproduction Science* 23: 69 (1-2): 15-23.

Dode M.A.N., Rodovalho N.C., Ueno V.G. and Alves R.G.O. (2001). Oocyte recovering from *Bos indicus* female. *Archives of Zoo technology* 50: 415-418.

Fortune J.E. (1994). Ovarian follicular growth and development in mammals. *Biology of Reproduction* 50: 225-232.

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

Hyttel P., Xu K.P. and Greve. T. (1988). Ultrastructural abnormalities of *in vitro* fertilisation of *in vitro* matured bovine ooctyes. *Anatomy and Embryology* 178: 47-52.

Islam F.M., Bhuiyan M.M.U., Banu T.A., Bhattacharjee J. and Shamsuddin M. (2007). *In vitro* maturation and fertilisation of oocytes in zebu cattle. *Bangladesh Veterinary Journal* 41 (1-4): 58-76.

Iwata H., Shiono H., Kon Y., Matsubara K., Kimura K., Kuwayama T. and Monji Y. (2008). Effects of modification of *in vitro* fertilisation techniques on the sex ratio of the resultant bovine embryos. *Animal Reproduction science* 105: 234-244.

Johnson A.E., Bormann C.L., Knee A.M., Swain J.E., Gibbons J.R. and Krisher R.L. (2001). Developmental competence of bovine oocyte retrieved from 5 and 10 mm follicles of a new follicular wave. *Theriogenology* (Abstract) 55: 405.

Kubisch H.M., Larson M.A., Funahashi H., Day B.N. and Roberts R.M. (1995). Pronuclear visibility, development and transgenic expression in IVM/IVF derived porcine embryos. *Theriogenology* 44: 391-401.

Leibfried-Rultedge M.L., Crister E.S., Eyestone W.H., Northey D.L. and First N.L. (1987/9). Developmental potential of bovine oocytes matured *in vitro* or *in vivo*. *Biology of Reproduction* 36: 376-383.

Leibfried-Rutledge M.L. (1999). Factors determining competence of *in vitro* produced cattle embryos. *Theriogenology* 49: 473-785.

Lightfoot D.A., Kouznetsova A., Mahdy E., Wilbertz J. and Hoog C. (2006). The fate of mosaic aneuploid embryos during mouse development. *Developmental Biology* 289: 384-394.

Lonergan P., Carolan C. and Mermillod P. (1994a). Development of bovine embryos *in vitro* following oocyte maturation under defined conditions. *Reproduction Nutrition Development* 34: 329–339.

Marquant-Le Guienne B., Humblot P., Thibier M. and Thibault C. (1993). Evaluation of bull semen fertility by homologous *in vitro* fertilisation test. *Reproduction Nutrition Development* 30: 259-266.

Mermillod P., Le Bourhis D., Lonergan P., Khatir H. and Heyman Y. (1998). Assessment of cytoplasmic competence of prepubertal calf oocytes by use of nuclear transfer. *Theriogenology* (Abstract) 49: 187.

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 J.J., Krogenaes A. and Susko-Parrish J.L. (1995). Effect of bovine sperm separation by either swim-up or percoll method on success of *in vitro* fertilisation and early embryonic development. *Theriogenology* 44: 859-869.

Parrish J.J., Susko-Parrish J.L., Winer M.A. and First N.L. (1988b). Capacitation of bovine sperm by heparin. *Biology of Reproduction* 38: 1171-1180.

Pavlok A., Lucas-Hahn A. and Niemann H. (1992). Fertilisation and developmental competence of bovine oocytes derived from different categories of antral follicles. *Molecular Reproduction and Development* 31: 63-67.

Rocha A., Randel R.D., Brousard J.R., Lim J.M., Blair R.M., Roussel J.D., Godke R.A. and Hansel W. (1998). High environmental temperature and humidity decrease oocyte quality in *Bos Taurus* not in *Bos indicus* cows. *Theriogenology* 49: 657-665.

Salamone D.F., Damiani P., Fissore R.A., Robl J.M. and Duby R.T. (2001). Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. *Biology of Reproduction* 64: 1761-1768.

Shamsuddin M. and Larsson B. (1993). *In vitro* development of bovine embryos after fertilisation using semen from different donors. *Reproduction in Domestic Animals* 28: 77-84.

Shamsuddin M., Rodriguez-Martinez H. and Larsson B. (1993c). Fertilising capacity of bovine spermatozoa selected after swim-up in hyaluronic acid containg medium. *Reproduction, Fertility and Development* 5: 307-315.

Staessen C., Janssenswillen P., Devroey P. and Van Steirteghen A.C. (1993). Cytogenetic and morphological observations of single pronucleated human oocytes after *in vitro* fertilisation. *Human Reproduction* 8(2): 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. *Animal Reproduction Science 48*: 187-195.

Suraeva N., Christjkov D. and Prokofiev M. (1997). The timing of pronuclear formation of IVM/IVF produced bovine zygotes and their development following microinjection. *Theriogenology* 47(1): 229-229.

Tanghe S., Van Soon A., Stercks V., Maes D., and Kruif A. de. (2002). Assessment of different sperm quality parameters to predict *in vitro* fertility of Bulls. *Reproduction in Domestic Animals* 37: 127-132.

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

Ward, F.A., Rizos D., Corridan D., Quinn K., Boland M.P. 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*. *Molecular Human Reproduction* 60: 47-55.

Xu K.P. and Greve T. (1988). A detailed analysis of early events during *in vitro* fertilisation of bovine follicular oocytes. *Journal of Reproduction and Fertility* 82: 127-134.

Zeitoun M.M., Rodriguez-Martinez H. and Randel R.D. (1996). Effects of season on ovarian follicular dynamics in Brahman cows. *Theriogenology* 45: 1577-1581.

Syrstad O. (1996). Dairy cattle crossbreeding in the tropics: Choice of crossbreeding strategy, *Tropical Animal Health and Production* Volume 28 (3): 223-229.

Photograph

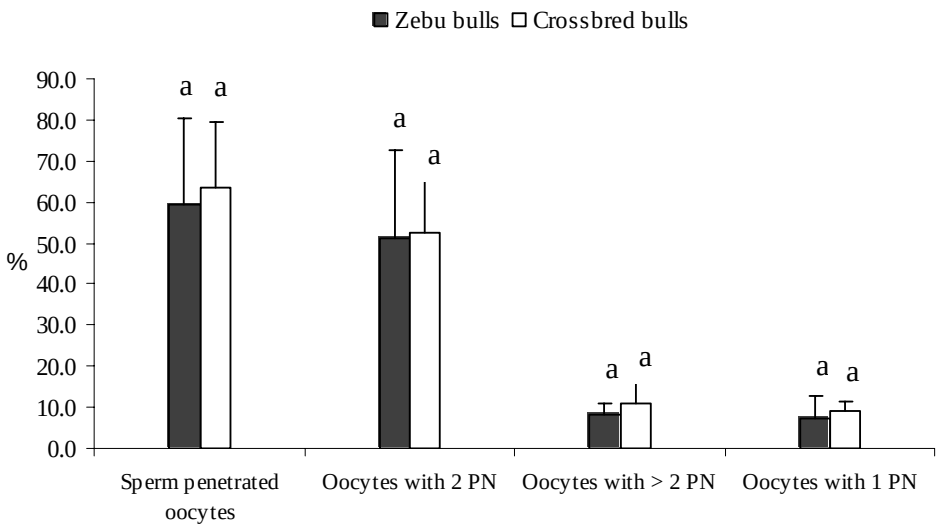

Figure 1. Mean ± SD percentages of oocytes penetrated by one or more spermatozoa, oocytes with two pronuclei, oocytes with more than two pronuclei and with one pronucleus 18 h after IVF with semen from two groups of bulls. Same letter above bars indicate statitically similar (P> 0.05). Number of bulls in each group = 5; the experiment was repeated 3 times with semen from each bull.

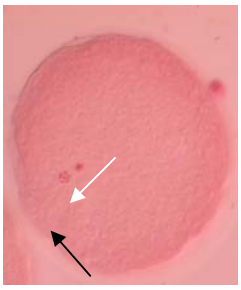

Figure 2. *In vitro* matured oocyte with metaphase II stage (black arrow) + polar body (white arrow)(1% aceto-orcein stain, DIC microscopy, 400 ×)

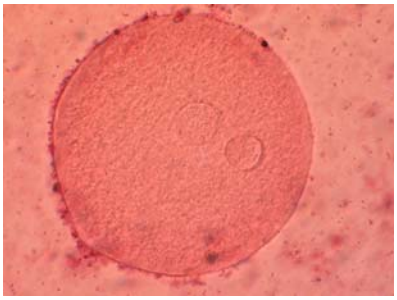

Figure 3. Normally fertilised oocyte (1% aceto-orcein stain, DIC microscopy, 400 ×)

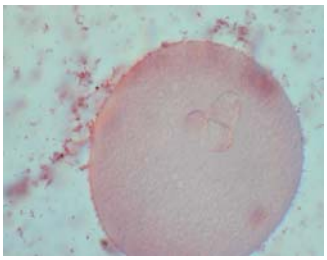

Figure 4. Polyspermic fertilisation (1% aceto-orcein stain, DIC microscopy, 400 ×)

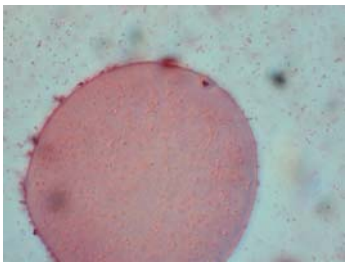

Figure 5. Oocyte with one pronucleus (1% aceto-orcein stain, DIC microscopy, 400 ×)