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Comparison of Culture Media for *In Vitro* Maturation of Oocytes of Indigenous Zebu Cows in Bangladesh

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ABSTRACT

The objectives of the present study were to select an effective basic medium including its hormone and protein supplementation for IVM of oocytes of indigenous zebu cows. The ovaries of cows were collected from slaughter house and the follicular fluid was aspirated from 2 to 8 mm diameter follicles. The COCs with more than 3 cumulus cell layers and homogenous cytoplasm were selected for maturation. The oocytes were matured in media for 24 hrs at 39℃ with 5% CO_2 in humidified air. The maturation of oocytes was evaluated by examining the presence of first polar body under microscope. An efficient basic medium was determined after culturing COCs in either TCM 199 or SOF medium in Experiment 1. An efficient hormone supplementation was determined after culturing COCs in either FSH or gonadotrophin supplemented TCM 199 in Experiment 2. An efficient protein supplementation was determined after culturing COCs in either FBS or Oestrous cow serum (OCS) supplemented TCM 199 in Experiment 3. The oocyte recovery rate per ovary was 3.35. The overall rate of IVM was 74.6%. The maturation rate was 75.5±3.9 and 62.2±20.2% in TCM and SOF medium, respectively (P>0.05). The maturation rate of oocytes was significantly higher (76.6±13.2%) in FSH supplemented medium than gonadotrphin supplemented counterpart (69.7±10.8%) (P<0.05). The maturation rates of oocytes were 81.7±12.9 and 85.7±12.7% in medium supplemented with FBS and OCS, respectively (P>0.05). In conclusions, both TCM 199 and SOF supplemented with either FBS or OCS, and FSH may be used as medium for IVM of indigenous zebu oocytes in Bangladesh.

(Key words: culture media, indigenous, IVM, oocytes, zebu cows)

INTRODUCTION

The indigenous zebu (*Bos indicus*) cattle of Bangladesh are lower yielding than the exotic (*Bos taurus*) cattle although the indigenous cattle are highly adapted to tropical environment and resistant to maximum diseases. There are mark deficit of milk and meat production in Bangladesh. Therefore, it is essential to upgrade the locally adapted indigenous zebu cattle for increasing milk and meat production. In Bangladesh, upgradation of indigenous zebu cows by artificial insemination using semen of exotic breed has been in practice for more than 4 decades (Ahmed and Islam, 1987). However, the progress of genetic up-gradation is slower than expectation. Therefore, it is essential for application of other assisted reproductive technologies (ARTs) for rapid production of crossbred cow to meet up the requirement of milk and meat.

Rapid generation of F_1 offspring can be done by applying ARTs such as multiple ovulation and embryo transfer (MOET) and *in vitro* embryo production (IVEP) followed by embryo transfer (ET) in zebu cows. The IVEP and ET is more suitable than MOET as IVEP can use slaughterhouse derived oocytes for generation of F_1 crossbred embryos. By this time, the IVEP and ET techniques have already been established with satisfactory results in many animals and humans in most of the country in the world. However, this IVEP-ET technique has not yet been established in this country.

In vitro maturation (IVM) of oocytes is the first and most important step for any IVEP-ET programme in cattle. However, although several preliminary studies have been conducted on IVM of oocytes of zebu cows in Bangladesh (Goswami, 2002,

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Rahman, 2003; Das *et al.*, 2006; Islam *et al.*, 2007; Talukder *et al.*, 2008; Morshed *et al.*, 2014) still the IVM rate needs to be improved. The maturation medium and supplementation of protein and hormones in it may play an important role for IVM rate and subsequent development after *in vitro* fertilization (IVF) (Bavister *et al.*, 1992). Therefore, the present study was conducted to improve the IVM rate of indigenous zebu cows oocytes in Bangladesh.

MATERIALS AND METHODS

The study was carried out at the Community based Dairy Veterinary Foundation (CDVF) Laboratory, Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh during the period from January to June, 2014.

1. Chemicals and Media

All the media constituents, reagents and chemicals were purchased from Sigma-Aldrich Inc., St Louis, USA. Media and reagents were prepared using standard protocol and under aseptic condition. All media were filtered using 0.22 μm pore size filter (Durapure® membrane filter, Carrigtwohill, Ireland) and culture medium was routinely equilibrated in incubator (VS-9000C, Vision Scientific Co. Ltd. South Korea) at 39℃ with 5% CO_2 in humidified air for at least 2 hrs before use.

2. Collection of Ovaries

The ovaries of indigenous zebu cows were collected from local slaughter house after slaughtering within 2 hrs (Fig. 1) and carried to the laboratory in a thermo flask containing warm normal saline (37℃, 0.9% sodium chloride solution, w/v).

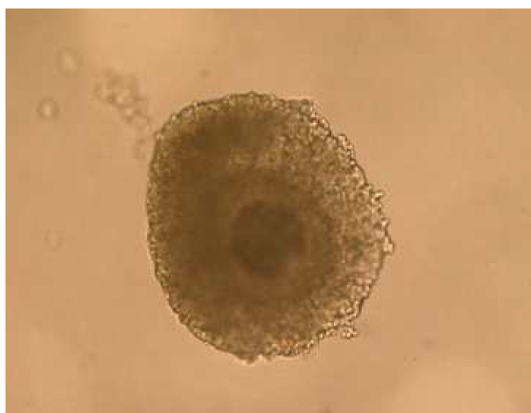

Fig. 1. An immature cumulus oocyte complex (COC) (10× objective).

3. Collection of Oocytes

In the laboratory, the ovaries were washed three times with warm (37℃) normal saline. The follicular fluid of 2 to 8 mm diameter follicles was aspirated using an 18 gauge needle (TERUMO®, Beijing, China) fitted with a 10 ml disposable plastic syringe (JMI Syringes and Medical Devices Ltd®, Chauddagram, Comilla, Bangladesh).

4. Selection of Occytes for Culture

The aspirated follicular fluid was transferred in a 60 mm petridish (Greiner bio-one, Frickenhausen, Germany) and left for 5 minutes for sedimentation. The retrieved follicular aspirate was diluted with HEPES-buffered tissue culture medium (TCM) 199 supplemented with bovine serum albumin (BSA) (washing medium) (Appendix 1). Oocytes (Fig. 1) were selected under a stereo-microscope (LABOMED, USA). The cumulus-oocyte-complexes (COCs) with more than 3 compact cumulus cell layers and homogenous ooplasm (Fig. 1) were selected for maturation. The COCs were washed three times in washing medium followed by once washing in maturation medium.

5. Culture of Oocytes for Maturation

Four 50 μl drops of maturation medium were prepared in 35 mm petridish (FALCON, Becton Dickinson Labware, USA) and covered with embryo tested mineral oil. For *in vitro* maturation, 7～10 COCs were cultured in each drop of medium in incubator at 39℃ with 5% CO_2 in humidified air for 24 hrs.

6. Evaluation of Oocytes for Maturation

The culture drops were examined under the stereo microscope for cumulus expansion after 24 hrs of culture in the maturation media. Presumptive maturation was confirmed by the degree of cumulus expansion (Fig. 2). To examine the presence of first polar body extrusion, the COCs were denuded by using denuding agent (3% sodium citrate, w/v in HEPES buffered TCM 199 medium) and pipetting. After pipetting, the denuded oocytes were kept in 10 μl drops of HEPES buffered TCM 199 and examined for presence of polar body under inverted microscope (Leica DM IRE2, Germany) with the help of a mouth controlled pipette (Fig. 3).

7. Experimental Design

1) Experiment 1

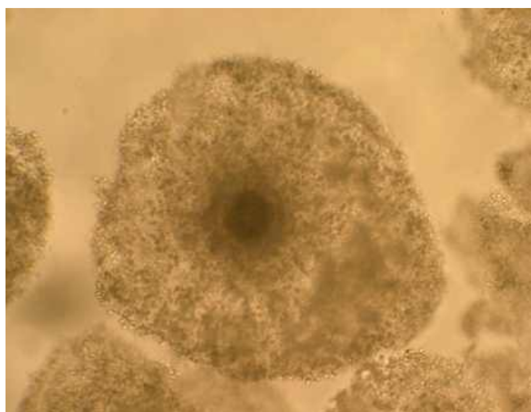

Fig. 2. An expanded COCs (10× objective).

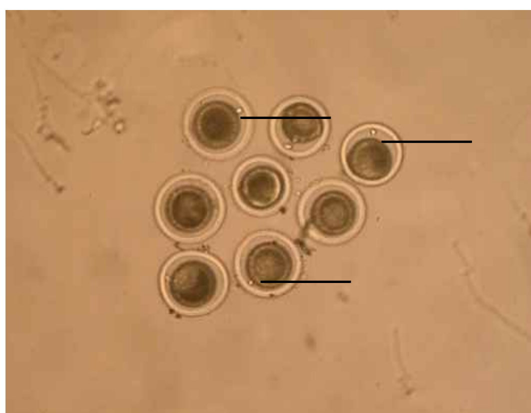

Fig. 3. Mature oocytes with polar body. Black arrow indicates polar body (10× objective).

To determine an effective basal maturation medium, the COCs were cultured either in TCM 199 (TCM, Earle's salts with sodium bicarbonate) (Appendix 2) or Synthetic oviductal fluid medium (SOF) (GIBCO, Invitrogen Corporation 3) supplemented with 10% fetal bovine serum (FBS), 5 μg/ml follicle stimulating hormone (FSH) and 1 μg/ml oestradiol (OE_2). Each experiment was repeated for 5 times across the days.

2) Experiment 2

To determine an effective hormone supplementation in maturation medium, the oocytes were cultured in TCM 199 supplemented with either 5 μg/ml FSH or 10 IU/ml gonadotrophin, 10% FBS and 1 μg/ml OE_2. Each experiment was repeated for 5 times across the days.

3) Experiment 3

To determine an effective protein supplementation in maturation medium, the COCs were cultured in TCM 199 supplemented with either 10% FBS or 10% OCS, 5 μg/ml FSH and 1 μg/ml OE_2. Each experiment was repeated for 6 times across the days.

8. Method of Oestrus Cow Serum (OCS) Preparation

Forty ml blood was collected from oestrus cow in a 50 ml sterilized plastic syringe containing no anticoagulant. Then the tube was kept in refrigerator at 4℃ in an upright position for 60 minutes to allow clotting. After clotting the tube was tilted and serum was collected in two 10 ml sterilized plastic tube. Then the serum containing tubes were centrifuged 15 minutes at 2,000 RPM for sedimentation of insoluble matter. After centrifugation the serum was inspected for turbidity. Then the serum was inactivated by heat in water bath at 56℃ for 30 minutes. After heat inactivation the serum was cooled in refrigerator at 4℃, and then the serum was aliquot of 500 μl in cryovials and store at −20℃ for further use.

9. Statistical Analysis

The data were recorded in Microsoft Excel spread sheet and descriptive statistics was performed. The maturation rates were expressed as mean±S.D. and the difference between groups was determined by Student's *t*-test. The difference between groups was considered significant when *P*-value was <0.05.

RESULTS

A total of 496 oocytes were collected from 148 ovaries and the mean number of oocytes collection from each ovary was 3.35. The overall maturation rate in the present investigation was 74.6% (206/276).

1. Experiment I: Determination of an Effective Basic Medium for Oocytes Maturation

The maturation rate of oocytes in two basic media is presented in Table 1. The maturation rates of oocytes were 75.5 ±3.9% and 62.2±20.2 in TCM 199 and SOF medium, respectively. However the maturation rate did not vary statistically between two media ($P>0.05$).

2. Experiment 2: Determination of an Effective Hormone Supplementation for Oocytes Maturation

The maturation rate of oocytes in two hormone supple-

Table 1. Effect of basic maturation media on IVM rate of zebu oocytes

Basic media used	Number of oocytes cultured	Number of oocytes matured	Maturation rate (%)
TCM 199	53	40	75.5±3.9
SOF	45	27	62.2±20.2

Number of replicates is 5. Proportion values are mean±S.D. The maturation rate was not significantly different from each other (P>0.05).

mented media is presented in Table 2. The maturation rate of oocytes was higher (76.6±13.2%) in FSH supplemented TCM 199 than that of gonadotrphin supplemented counterpart (69.7 ±10.8%). The difference in maturation rate was statistically significant between two hormone supplementations (P<0.05).

3. Experiment 3: Determination of an Effective Protein Supplementation for Oocytes Maturation

The maturation rate of oocytes in two protein supplemented media is presented in Table 3. The maturation rates of oocytes were 81.7±12.9% in medium supplemented with FBS and 85.7 ±12.7 in OCS, respectively. However the maturation rate did not vary statistically between two protein supplementations (P>0.05).

DISCUSSION

The objectives of the present study were to select an effective basic medium and its effective hormone and protein supplementation for IVM of oocytes of indigenous zebu cows. In the present study, the mean number of oocytes collection from each ovary was 3.35. The present mean number of retrieved oocytes per ovary is higher than that of previous study

Table 2. Effect of hormone supplementation on IVM rate of zebu oocytes

Hormone supplementation	Number of oocytes cultured	Number of oocytes matured	Maturation rate (%)
FSH	49	38	76.6±13.2[a]
Gonadotrophin	39	27	69.7±10.8[b]

Number of replicates is 5. Proportion values are mean±S.D.
[a,b] The values with superscripts within same column was significantly different from each other (P<0.05).

Table 3. Effect of protein supplementation on IVM rate of zebu oocytes

Protein supplementation	Number of oocytes cultured	Number of oocytes matured	Maturation rate (%)
FBS	45	36	81.7±12.9
OCS	45	38	85.7±12.7

Number of replicates is 6. Proportion values are mean±S.D. The maturation rate was not significantly different from each other (P>0.05).

(Morshed *et al.*, 2014) and lower than that of an earlier study (Talukder *et al.*, 2008). The reason for variations in oocyte retrieval rate among studies may be due to variation in skill of follicle aspirators. Moreover, seasons of oocytes retrieval and cyclic status of cows may influence the oocyte retrieval from ovaries (Dode and Adona, 2001). Additionally, an increase in FSH level in blood may influence the number of oocytes retrieved (Fortune, 1994). Further, nutrition and temperature may influence the gonadotrophin concentrations and affect the population of follicles and number of oocytes retrieved (Zeitoun *et al.*, 1996).

In the present study, the rate of overall oocyte maturation was 74.5%. The present maturation rate is similar to the earlier study reported by Talukder *et al.* (2008) in indigenous zebu cows. However, contrasting to the present findings, lower maturation rate was reported by Das *et al.*, (2006) (65.4%) and Morshed *et al.*, (2014) (53.8%) in indigenous zebu cows. The reasons for variation in maturation rate among studies might be due to variation in basic media and percentage of serum supplementation in it used for oocyte maturation. Moreover, grades of oocytes may influence the *in vitro* maturation rates of oocytes as variation in rate of maturation *in vitro* was demonstrated between good and poor grade oocytes (Goswami, 2002). However, all retrieved oocytes were cultured for maturation irrespective of grading which may contribute for obtaining lower maturation rate by Morshed *et al.* (2014) than that of present study. In the present study, oocytes with at least 3 compact cumulus cell layers were used for maturation which might contribute to obtaining satisfactory rate of oocyte maturation *in vitro*.

For maturation of oocytes *in vitro*, ingredients of a culture media play an important role. Different culture media such as TCM-199 (Kharche, 2006; Amer *et al.*, 2008), SOF (Totey *et al.*, 1992), minimum essential medium (MEM) (Ravindranatha,

2001) and Ham's F-10 (Totey et al, 1993; Tamilman *et al.*, 2005) have been used for IVM of mammalian oocytes elsewhere. Among them, TCM 199 is the most widely used culture medium for such purposes (Arunakumari *et al.*, 2007). The beneficial effect of TCM-199 medium on IVM of animal oocytes may be attributed due to presence of some factors in its composition such as essential amino acids and glutamine that may stimulate DNA and RNA synthesis and enhance cell division (Pawshe *et al.*, 1996; Gordon, 2003). Moreover, there is a report that TCM 199 improved the rate of IVM of oocytes better than MEM in buffaloes (Roushandeh et al, 2006). It has been known for many years that glucose and glutamine are poor energy substrates for the cumulus cell- free rodent oocytes (Downs and Verhoeven, 2003). The lower IVM rate in MEM may be explained by the fact that it contains higher glucose and glutamine than that of TCM 199. In the present investigation, the maturation rate of oocytes did not vary between oocytes cultured in either TCM 199 or SOF ($P>0.05$). Similar to the present study, Prasad *et al.* (2013) did not obtain any difference in IVM rates when maturation rate of buffalo oocytes were compared between TCM 199 and SOF. Contrasting to the present study, Totey *et al.* (1992) reported higher percentage of oocytes maturation in TCM than that of SOF in buffaloes. However, when compared the rate of cleavage and blastocyst formation after IVF, there were no difference in cleavage and balstocyst formation in bovine oocytes matured either in defined TCM 199 or SOF (Lonergran *et al.*, 1994). The reasons for variations in IVM rate among studies may be due to variation in supplementations in basic media.

In the present study, the maturation rate of oocytes was significantly ($P<0.05$) higher in FSH supplemented TCM 199 than that of gonadotrphin supplemented counterpart. Contrasting to the present study, although IVM rate was not compared, the embryo development rate after IVF did not vary between bovine oocytes cultured either in FSH or gonadotrophin supplemented medium. Moreover, the IVM rate did not vary in buffalo oocytes matured in either FSH or PMSG supplemented medium (Hegab *et al.*, 2009). Further, no difference in IVM rate of bovine oocytes was observed when compared between FSH and LH supplementation (Younis *et al.*, 1989). Supplementation of reproductive hormones in maturation media is essential because it improves IVM rate of mammalian oocytes. Addition of follicle stimulating hormones (FSH) (Chauhan *et al.*, 1998), pregnant mare serum gonadotrophin (PMSG) (Roy *et al.*, 1968), luteinizing hormone (LH) and oestradiol (Nandi *et al.*, 2002b) to maturation media has been made to improve the developmental competence of *in vitro* matured oocytes. In many mammalian species, gonadotrophin has been found to stimulate cumulus cells to synthesize molecules able to drive germinal vesicle breakdown (GVBD) as meiosis activating sterols (Tsafriri *et al.*, 2005). Oestradiol has been found to improve the completion of maturational changes and also to support the synthesis of presumed male pronuclear growth factor (Fukui and Ono, 1989). Moreover, FSH is essential for cumulus cell expansion and maturation of oocytes *in vitro* as FSH enhances the expansion of cumulus cells in buffaloes (Alok *et al.*, 2010). Nevertheless, in addition to either FSH or gonadotrophin, oestradiol was always supplemented in maturation medium in the present study.

In the present study, the maturation rate of oocytes did not vary in OCS supplemented TCM 199 than that of FBS supplemented counterpart ($P>0.05$). Contrasting to the present finding, Lu and Gordon (1987) reported that OCS had a significant and marked effect on oocytes maturation compared to FBS supplementation. Further, similar result to earlier report has been demonstrated elsewhere (Schellander *et al.*, 1990). Serum may provide energy substrates, amino acids, growth factors and vitamins to the culture medium. The positive effect of serum on maturation rate of oocytes may be due to presence of growth factors that is manifested by improved embryo development following IVF (Eppig *et al.*, 1992). Moreover, it is important to include serum in the IVM medium to prevent hardening of zona pellucida which could adversely affect fertilization (Downs *et al.*, 1986). Additionally, there is a report that fetuin, a major glycoprotein constituent of fetal calf serum, can prevent hardening of zona pellucida during IVM (Schroeder *et al.*, 1990). Kan and Yamane (1983) reported another beneficial action of serum due to its antioxidant properties as evidenced by reducing superoxide formation. In addition, serum added to the maturation medium provides a source of albumin that balances the osmolarity (Thompson, 2000).

There are different sources for supplemented sera such as FBS (Kobayeshi *et al.*, 1994; Nandi, 1998; Mahmoud and Nawito, 2005; Das *et al.*, 2006; Talukder *et al.*, 2008), OCS (Schellander *et al.*, 1990), steer serum (Roy *et al.*, 1968; Nandi *et al.*, 2001), and super ovulated cow serum (Boediono *et al.*, 1994). Although, there are reports to use different sera sources for supplementation in maturation medium, comparison was

made between FBS and OCS only in the present study. Because, FBS and OCS are the most widely used protein supplementation for maturation culture of bovine oocytes in the world.

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