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DETERMINATION OF AN EFFECTIVE MEDIA AND ITS HORMONE AND PROTEIN SUPPLEMENNTATION FOR *IN VITRO* MATURATION OF OOCYTES OF INDIGENOUS ZEBU COWS

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ABSTRACT

In vitro maturation (IVM) of oocytes is the first important step for successful in vitro embryo production of any mammalian species. The objectives of the present study were to determine an effective basic medium and its hormone and protein supplementation for IVM of oocytes of indigenous zebu cows. The oocytes were derived from ovaries of locally slaughtered cows after aspiration of follicle. The oocytes were cultured in medium for 24 hrs at 38.5ºC with 5% CO_2 in humidified air for maturation. The maturation of oocytes was evaluated by examining the presence of first polar body extrusion in denuded oocytes under inverted microscope. To determine an effective basic medium, the oocytes were cultured in fetal bovine serum (FBS) supplemented tissue culture medium (TCM), modified synthetic oviduct fluid (mSOF) and Tyrodes albumin lactate pyruvate (TALP) medium. The maturation rate was significantly higher (74±4.2) in TCM medium than that of TALP medium (58.2±6.2). To determine an effective hormone supplementation for maturation medium, the oocytes were cultured in either in follicle stimulating hormone (FSH) or gonadotrophin supplemented TCM. The maturation rate of oocytes was significantly ($p>0.05$) higher (73.3±4.0) in FSH supplemented medium than that of gonadotrophin supplemented counterpart (60.2±6.6). To determine an effective protein supplementation, the oocytes were cultured in FBS, oestrus cow serum (OCS) and bovine serum albumin (BSA) supplemented TCM 199. The maturation rate of oocytes were 73.0±5.9, 71.1±2.8, and 62.5±9.4 in medium supplemented with FBS, OCS and BSA respectively ($p>0.05$). In conclusions, TCM supplemented with either FBS, OCS or BSA as protein and FSH as hormone may be used as medium for IVM of oocytes of indigenous zebu cows.

Keywords: Hormone, IVM, Media, Oocytes, Protein, Zebu cows

INTRODUCTION

Livestock plays a major role in the life of farmers in developing countries. It provides food, income, employment and many other contributions including production of hide, draft power, fertilizer and fuel. Bangladesh is highly density populated country in the world. There are mark deficit of milk and meat production in Bangladesh. 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. Therefore, it is essential to upgrade the locally adapted indigenous zebu cattle for increasing milk and meat production. The up-gradation of indigenous zebu cows by artificial insemination using semen of exotic breed has been in practice for more than 4 decades in Bangladesh (Ahmed and Islam, 1987). However, the progress of genetic up-gradation is slower than expectation. So it is essential for application of other assisted reproductive technologies (ARTs) for rapid production of crossbred cattle to meet up the requirement of milk and meat.

Rapid generation of F1 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 F1 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 (Das *et al*., 2006; Islam *et al*., 2007; Talukder *et al*., 2008; Morshed *et al*., 2014; Singha *et al*., 2015; Choudhury *et al*., 2017) 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 select an efficient basic medium including supplementation of suitable hormone and protein in it for optimal rate IVM of oocytes of indigenous Zebu cows in Bangladesh.

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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.

Chemicals and media

All the chemicals, reagents and media constituents 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 0.22µm 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 38.5°C with 5% CO_2 in humidified air for at least 2 hrs before use.

Collection of ovaries

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

Collection of oocytes

In the laboratory, the ovaries were rinsed three times with warm (37°C) 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)

Selection of oocytes 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). Oocytes were selected under a stereo-microscope (LABOMED, USA). The cumulus-oocyte-complexes (COCs) with more than 3 compact cumulus cell layers and homogenous ooplasm were selected for maturation. The COCS were washed three times in washing medium followed by once washing in maturation medium.

Culture of oocytes for maturation

Four 50µl drops of maturation medium were prepared in 35mm petridish (FALCON, Becton Dickinson Labware, USA) and covered with embryo tested mineral oil. For *IVM*, 8-10 COCs were cultured in each drop of medium in incubator at 38.5°C with 5% CO_2 in humidified air for 24 hrs.

Evaluation of oocytes for maturation

The culture drops were examined under the stereo microscope for expansion of cumulus cell after 24 hrs of culture in the maturation media. Presumptive maturation was confirmed by the degree of cumulus expansion. 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 10µl 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.

Experimental design

Experiment 1

To determine an effective basal maturation medium, the COCs were cultured in TCM 199 (TCM, Earle's salts with sodium bicarbonate), modified synthetic oviductal fluid (mSOF) medium and Tyrodes albumin lactate pyrovate (TALP) medium supplemented with 10% fetal bovine serum (FBS), 5µg/ml follicle stimulating hormone (FSH) and 1µg/ml oestradiol (OE_2). Each experiment was repeated for 10 times across the days.

Experiment 2

To determine an effective hormone supplementation in maturation medium, the oocytes were cultured in TCM 199 supplemented with 5µg/ml FSH or 10 IU/ml Gonadotrophin, 10% FBS and 1µg/ml OE_2. Each experiment was repeated for 8 times across the days.

Experiment 3

To determine an effective protein supplementation in maturation medium, the COCs were cultured in TCM 199 supplemented with 10% FBS, 10% OCS (Oestrous Cow Serum) or 3% BSA (Bovine serum albumin) and 5µg/ml FSH and 1µg/ml OE_2. Each experiment was repeated for 8 times across the days.

Statistical analysis

The data were recorded in Microsoft Excel spread sheet and descriptive statistics was performed. The maturation rates were expressed as mean ± SD and Z test was done to determine the significant effect of different treatments on the maturation rate using SPSS Version 20. The difference between groups was considered significant when P-value was < 0.05.

RESULTS AND 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. A total of 2340 oocytes were collected from 585 ovaries and the mean number of oocytes collection from each ovary was 4.00. The present mean number of retrieved oocytes per ovary is higher than that of previous study (Morshed *et al.*, 2014; Singha *et al*., 2015; Choudhury *et al*., 2017) 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 skillness of follicle aspirators. Moreover, seasons of oocytes retrieval and cyclic status of cows may influence the oocyte retrieval rate 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 67.6% (785/1161). The present maturation rate is lower than previous study reported by Singha *et al.* (2015) in indigenous zebu cows. However, contrasting to the present findings, lower maturation rate was reported by Morshed *et al.* (2014) (53.8%) and Das *et al*. (2006) (65.4%) 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.*

Experiment I. Determination of an effective basic medium for oocytes maturation

The maturation rate of oocytes in three basic media is presented in Table 1. The maturation rates of oocytes were 74.5 ± 4.2%, 67.7 ± 5.8% and 58.2±6.2 in TCM 199, mSOF and TALP medium, respectively. The deference in maturation rate was significant between TCM and TALP media (P<0.05).

Table1. Effects of basic maturation media on IVM rate of Zebu oocytes

Basic medium	Number of oocytes cultured	Number of oocytes matured	Maturation rate (%)
TCM	145	108	74.5 ± 4.2^{a}
mSOF	133	90	67.7 ± 5.8^{ab}
TALP	134	78	58.2 ± 6.2^{b}

Number of replicates is 10. Proportion values are mean ± SD.

[a,b] The values with superscripts within same column were significantly different from each other (P<0.05).

For maturation of oocytes *in vitro*, ingredients of a culture media play an important role. Different culture media such as TCM-199 (Kharche *et al*., 2006; Amer *et al.*, 2008), SOF (Totey *et al.*, 1992), minimum essential medium (MEM) (Ravindranatha *et al*., 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 was significantly higher in TCM than TALP media but did not vary with SOF medium ($P > 0.05$). 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.

Experiment 2. Determination of an effective hormone supplementation for oocytes maturation

The maturation rate of oocytes in two hormone supplemented media is presented in Table 2. The maturation rate of oocytes was higher (73.3 ± 4.0%) in FSH supplemented TCM 199 than that of gonadotrphin supplemented counterpart (60.2 ± 6.6%). The difference in maturation rate was statistically significant between two hormone supplementations ($P<0.05$).

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

Hormone supplementation	Number of oocytes examined	Number of oocytes matured	Maturation rate (%)
FSH	161	118	73.3 ± 4.0^{a}
Gonadotrophin	161	97	60.2 ± 6.6^{b}

Number of replicates is 8. Proportion values are mean ± SD.
[a,b] The values with superscripts within same column were significantly different from each other ($P<0.05$).

In IVM, gonadotropins are the main stimulator of the oocyte development and FSH being deemed vital for the oocytes becomes qualified to be *in vitro* fertilized (Lu *et al.*, 2014; Khan *et al*., 2015). 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 (Groza *et al.*, 2008). 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 FSH (Chauhan *et al.*, 1998), PMSG (Roy *et al.*, 1968), LH and oestradiol (Nandi *et al.*, 2002) 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 it enhanced 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.

Experiment 3. Determination of an effective protein supplementation for oocytes maturation

The maturation rate of oocytes in three protein supplemented media is presented in Table 3. The maturation rates of oocytes were 73.0 ± 5.9%, 71.1± 2.8 and 62.5 ± 9.4 in medium supplemented with FBS, OCS and BSA, respectively. However the maturation rate did not vary among three protein supplementations ($P > 0.05$).

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

Protein supplementation	Number of oocytes examined	Number of oocytes matured	Maturation rate (%)
FBS	141	103	73.0 ± 5.9
OCS	142	101	71.1 ± 2.8
BSA	144	90	62.5 ± 9.4

Number of replicates is 8. Proportion values are mean ± SD.
The maturation rate was not significantly different from each other (P > 0.05).

There are several known functions of protein in culture medium. 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). Albumin chelates heavy metals and provides some p^H buffering, which can also be achieved by inclusion of amino acids in culture medium (Mehta and Kiessling, 1990). The surfactant property of albumin prevents adhesion of cells to glass and plastic surface (Pinyopummintr and Bavister, 1991) and also can act as a scavenger of reactive oxygen species (Natsuyama *et al*., 1993). Protein supplements during IVM medium can have profound effects both on the rate of development and on the overall efficiency of development shown by the morula and blastocyst yields. (Atef ali and Marc- Andre Sirard, 2002). 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).

In the present study, the maturation rate of oocytes did not vary in OCS supplemented TCM 199 than that of FBS or BSA 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). 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; Singha et al., 2015), steer serum (Roy *et al.*, 1968; Nandi *et al.*, 2001) and superovulated cow serum (Boediono *et al.*, 1994). Although, there are reports to use different sera sources for supplementation in maturation medium, comparison was made within FBS, OCS and BSA only in the present study. Because, FBS, OCS and BSA are the most widely used protein supplementation for maturation culture of bovine oocytes in the world.

In conclusions, selection of a suitable oocytes culture medium is crucial for optimal results in bovine IVM/IVF. For IVM, both TCM and mSOF may be used as a basic medium supplemented with FBS, OCS or BSA as protein source and FSH may be used as a hormone supplement in basic medium for optimum IVM rate of indigenous Zebu cow's oocytes in Bangladesh.

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