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Culture Conditions for *In Vitro* Maturation of Abattoir Derived Oocytes of Native Zebu Cows of Bangladesh

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ABSTRACT

The objectives of the study were to determine an effective culture dish, culture duration and protein supplementation in medium for *in vitro* maturation (IVM) of oocytes of native zebu cows in Bangladesh. The ovaries of cows were collected from local slaughterhouse followed by aspiration of follicular fluid. The cumulus-oocyte-complexes (COCs) with more than 3 compact cumulus cell layers were cultured in tissue culture medium (TCM) 199 for maturation. The maturation of oocytes was determined by observing polar body under microscope. To determine an effective culture dish, 130 COCs derived from 48 ovaries in a well of 4-well dish and 102 COCs derived from 36 ovaries in drops covered with mineral oil within 35 mm petri dish were cultured for 24 hours. The rate of maturation of oocytes did not vary between 4-well dish (51.3 ± 15.0 %) and drops in petri dish (52.4 ± 11.6 %). To determine the effective culture duration, 185 COCs derived from 62 ovaries were cultured in drops for 18, 21, 24 and 27 hours. The rate of maturation of occytes ranged from 51.9 ± 9.4 % (18 hours) to 59.0 ± 17.1 % (27 hours) and the difference in maturation rate among different culture durations was not significant (P>0.05). To determine an effective protein supplementation, 63 oocytes from 19 ovaries were cultured separately in TCM 199 supplemented with either fetal bovine serum (FBS) or bovine serum albumin (BSA). The rate of maturation was significantly (P<0.01) higher in medium supplemented with FBS (55.63 ± 16.19 %) than that of BSA (14.82 ± 9.36 %). In conclusion, COCs of native zebu cows can be cultured for IVM either in 4-well culture dish or droplets in petri dish for 18 to 27 hours in medium supplemented with FBS.

(Key words : *in vitro* maturation, oocytes, culture conditions, zebu cows)

INTRODUCTION

Tropically adapted nondescript zebu (*Bos indicus*) cows of Bangladesh are low yielding ones with comparison to exotic (*Bos traurus*) cows although the latter is poorly adapted in tropical environmental condition like Bangladesh. Therefore, it is essential to upgrade the locally adapted zebu cows for increasing milk production. Keeping this in mind, the Department of Livestock Services of Bangladesh introduced artificial insemination (AI) using semen of exotic breeds for cross breeding native cows since 1958 (Ahmed and Islam, 1987). However, indiscriminate use of semen from exotic breeds without adapting any breeding policy in Bangladesh resulted in many crossbred cows with unknown genetics. It has been demonstrated that cows resulting from crossbreeding (F_1 generation) possess both adaptation and production potential including expressing heterosis (Cunningham and Syrstad, 1987). However, it has also been demonstrated for years that daughters of F_1 cows and subsequent generations are not productive or not adaptive or both under tropical conditions (Syrstad, 1989). This indicates that the subsequent generations originated from F_1 cross breeds will make little contribution to the increased demand of milk production in tropical country like Bangladesh. This emphasizes the rapid generation of F_1 crossbred population of cows for maximum milk production in Bangladesh.

Rapid generation of F_1 offspring can be done by applying assisted reproductive technologies (ARTs) such as multiple ovulation and embryo transfer (MOET) and *in vitro* fertilization (IVF) of IVM oocytes followed by embryo transfer (ET) in zebu cows. Applying IVM-IVF-ET is more advantageous than

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that of MOET as IVM-IVF-ET can use abattoir derived oocytes for generation of transferable F_1 embryos. Accordingly, the IVM-IVF-ET techniques have already been established in many animals in most of the countries in the world for rapid generation of animals with desired traits. The IVM of oocyte is the first step towards the successful production of embryos *in vitro*. However, limited studies have been done on IVM of oocytes of zebu cows in Bangladesh (Goswami, 2002; Rahman, 2003; Das *et al.*, 2006; Islam *et al.*, 2007; Talukder *et al.*, 2008). Moreover, the IVM rate of oocytes may be influenced by the dish of oocyte culture, duration of IVM culture (Dode and Adona, 2001) and types of protein supplementation in culture media (Rose *et al.*, 1992; Kobayashi *et al.*, 1994; Holm *et al.*, 1999; Alm *et al.*, 2008). Therefore, the present study was conducted to determine an efficient culture dish, culture duration and protein supplementation in medium for IVM of oocytes of native zebu cows in Bangladesh.

MATERIALS AND METHODS

The study was carried out at the laboratory of Community-based Dairy Veterinary Foundation (CDVF), Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh during the period from June to October, 2013.

1. Chemicals and Media

All the chemicals, reagents and media constituents were purchased from Sigma-Aldrich Chemicals, St Louis, USA. Media and reagents were prepared using standard protocol following aseptic technique. The final media for washing and maturation culture were filtered using 0.22 μm pore size filter (Durapure® membrane filter, Carrigtwohill, Ireland).

2. Collection of Ovary

The ovaries of native zebu cows were collected from local slaughterhouse and carried to the laboratory in a thermo flask containing warm normal saline (37℃, 0.9% sodium chloride solution, w/v) within 2 hours of slaughtering.

3. Collection of Oocytes

In the laboratory, the ovaries were rinsed 3 times in normal saline at 37℃. The follicular fluid from 2 to 8 mm diameter follicles was aspirated using an 18 gauge needle (TERUMO®, Beijing, China) fitted with a 10 ml disposable plastic syringe (Opsosaline®, Dhaka, Bangladesh).

4. Oocytes Selection for Culture

The follicular fluid was transferred in a 60 mm petri dish (Greiner bio-one, Frickenhausen, Germany) and diluted with HEPES-buffered TCM 199 supplemented with BSA. The COCs with more than 3 compact cumulus cell layers were selected under a stereo microscope (Leica Microsystems, MZ6, Wetzlar, Germany).

5. Culture of Oocytes for Maturation

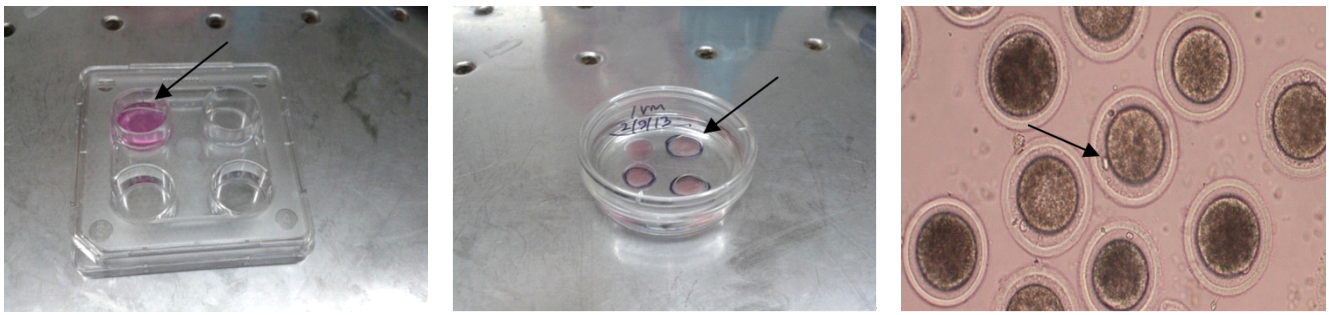
TCM-199 (With Earle's salts, L-glutamine and sodium bicarbonate) was the basic media for oocyte maturation. Bicarbonate buffered TCM 199 supplemented with 0.011 mg/ml sodium pyruvate, 5 mg/ml FSH, 1 mg/ml estradiol and 0.01 ml/ml penicillin-streptomycin solution was used for maturation. Moreover, as protein supplement, the culture medium was supplemented either with 10% FBS (v/v) or 5 mg/ml BSA according to the experimental design. The COCs were washed 3 times in washing TCM 199 followed by washing once in culture media before being cultured. The COCs were cultured in incubator at 39℃ in presence of 5% CO_2 in a humidified air following the method described elsewhere (Bhuiyan *et al.*, 2004). According to the experimental design, COCs were cultured either in 4-well dish (Nunclon, Rosklide, The Netherlands) or in droplets in 35 mm petri dish (Greiner bio-one, Frickenhausen, Germany). Thirty to forty COCs were cultured in 500 μl media in single well of a 4-well dish (Fig. 1A) and 8～12 COCs were cultured in mineral oil covered 50 μl droplets of media in a 35 mm petri dish (Fig. 1B). The duration of culture varied from 18 to 27 hours according to experimental design.

6. Evaluation of Oocytes for Maturation

The wells of 4-well dish and droplets of petri dish were examined under the stereo microscope for cumulus expansion after maturation culture. The maturation of oocytes was determined by observing extrusion of first polar body within denuded oocytes under inverted microscope (Fig. 1C). The COCs were denuded by vortexing for 2 minutes in presence of 3% sodium citrate (w/v) in HEPES buffered TCM 199 followed by pipetting with mouth controlled pipette.

7. Experimental Design and Statistical Analysis

In Experiment 1, COCs were cultured either in well of 4-



(A) (B) (C)

Fig. 1. (A) A 4-well dish used for IVM of occytes (arrow indicates culture media in a well), (B) A petri dish used for IVM of oocytes (arrow indicates droplet of culture media) and (C) denuded oocytes after maturation culture (arrow indicates extruded polar body in periviteline space of a mature oocyte).

well dish or droplets in 35 mm petri dish for 24 hours for determination of an effective culture dish for IVM. In Experiment 2, COCs were cultured in droplets in 35 mm petri dish for 18, 21, 24 and 27 hours for determination of optimum duration of culture for IVM. In Experiment 3, COCs were cultured in droplets in 35 mm petri dish for 24 hours in media supplemented with either FBS or BSA for determination of an effective protein supplementation in culture media. Each experiment was repeated at least 3 times across the days.

The data were entered in Microsoft Excel spread sheet and descriptive statistics was performed. The maturation rates were expressed as mean ± SD and the difference between groups was determined by Chi-square test. The difference between groups was considered significant when the *P* value was <0.05. All the analysis was performed using SPSS software.

RESULTS

The follicles from 165 ovaries were aspirated and 480 oocytes were retrieved. The mean number of oocytes retrieved from each ovary was 3.0 ± 0.2. The rate of overall maturation was 53.8 ± 2.7%. The matured oocytes showed a clear expansion of cumulus cells and extrusion of the first polar body.

1. Experiment 1 : Determination of an Effective Culture Dish

The IVM rate with respect to two types of culture dishes is presented in Table 1. The rate of maturation of oocytes was 51.3 ± 15.0% when COCs were cultured in 4-well dish and the maturation rate was 52.4 ± 1.6% when the COCs were cultured in droplets in petri dish. However, the difference in maturation rate did not vary between 2 types of culture dishes.

Table 1. Effect of culture dish on IVM rate of oocytes of local zebu cows

Type of culture dish	No. of oocytes examined	No. of replicates	Rate of maturation (%)
Culture in well of 4-well dish	96	4	51.3 ± 15.0%
Culture in drops within petri dish	45	3	52.4 ± 11.6%

Percentage values are mean ± S.D. The percentage values within same column did not differ significantly from each other (P>0.05).

2. Experiment 2: Determination of Effective Culture Duration

The IVM rate of oocytes of local zebu cows with respect to culture duration is presented in Table 2. The rate of maturation of oocytes was the lowest (51.9 ± 9.4%) when the COCs were cultured for 18 hours and the maturation rate was the highest (59.0 ± 17.1%) when the COCs were cultured for 27 hours. However, the difference in maturation rate of oocytes did not vary significantly among different duration of culture period.

3. Experiment 3: Determination of an Effective Protein Supplementation

The IVM rate of oocytes of zebu cows with respect to protein supplementation in culture media is presented in Table 3. The rate of maturation of oocytes was higher when the medium was supplemented with FBS (55.63 ± 16.19 %) than that of media supplemented with BSA (14.82 ± 9.36 %). The difference in rate of maturation between 2 types of protein supplementation was statistically significant (P<0.05).

Table 2. Effect of culture duration on IVM rate of oocytes of local zebu cows

Culture duration (hr)	No. of oocytes examined	No. of replicates	Rate of maturation (%)
18	38	5	51.9 ± 9.4
21	42	5	52.6 ± 10.7
24	45	6	55.5 ± 19.5
27	29	5	59.0 ± 17.1

Percentage values are mean ± S.D. The percentage values within same column did not differ significantly from each other (P>0.05).

Table 3. Effect of protein supplementation in culture medium on IVM rate of oocytes of local zebu cows

Types of protein supplementation	No. of oocytes examined	No. of replicates	Rate of maturation (%)
Fetal bovine serum (FBS)	27	3	55.6 ± 16.2[a]
Bovine serum albumin (BSA)	27	3	14.8 ± 9.3[b]

Percentage values are mean ± S.D.
[a,b] The values with superscripts within same column differed significantly from each other (P<0.01).

DISCUSSION

The first step towards *in vitro* production of embryo is oocytes maturation. In this study, attempts were taken to optimize the culture conditions for immature oocytes from slaughter house ovaries of zebu cows. In this study, the follicles from 165 ovaries were aspirated and 480 oocytes were retrieved where mean number of oocytes retrieved from each ovary was 3.0. However, number of retrieved oocytes per ovary was higher than that of present study in earlier investigations (Goswami 2002; Das *et al.*, 2006; 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 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 maturation was 53.8 %. Contrasting to the present finding, the *in vitro* maturation rate of oocytes of zebu cows was higher in Bangladesh (Das *et al.*, 2006; Talukder *et al.*, 2008) and Brazil (Dode and Adona, 2001) in earlier studies. 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 in the present study than earlier studies.

In the present study, the culture dish did not affect the *in vitro* maturation rate of oocytes as indicated by absence of any significant difference in maturation rate between oocytes cultured in drops of petri dish or well of 4-well dish. However, no previous investigation on effect of culture dish is evident on zebu cows elsewhere. In the present study, droplet of culture media was covered by mineral oil which could affect maturation of occytes *in vitro*. Because, used oestradiol in culture media may be absorbed by mineral oil resulting in reduced rate of maturation *in vitro* (Armstrong *et al.*, 1996).

In the present study, the culture duration did not affect the *in vitro* maturation rate of oocytes as indicated by absence of any significant difference in maturation rate among oocytes cultured for different durations. Semple *et al.* (1993) reported that bovine oocytes achieved developmental competency within 14 hours of commencing maturation *in vitro* and checked maturation at 18 hours of duration. Contrasting to the present finding, Dode and Adona (2001) reported significantly higher rate of maturation in 24 hours culture duration than that of 18 and 21 hours counterparts in zebu cows of Brazil. The reason for variation in maturation rates between studies may be due to variation in laboratory procedures and medium.

When timing of nuclear events during maturation *in vitro* was evaluated, the germinal vesicle (GV) was evident from 0 to 6.6 hours, GVBD occurred at 6.6～8.0 hours, chromatin condensation at 8～10.3 hours, metaphase I at 10.3～15.4 hours, anaphase I at 15.4～16.6 hours, telophase I at 16.6～18.0 hours and metaphase II at 18.0～24.0 hours (Sirard, 1989). Enright *et al.* (2000) investigated the effect of duration of maturation and concluded that maturation should be carried out for 24

hours. Moreover, partially denuded oocytes, totally denuded oocytes and oocytes with expanded cumulus cells at the starting of maturation culture progressed to metaphase II faster than compact cumulus-oocyte-complexes which may influence the rate of maturation (Spiropoulos and Long, 1989). However, in the present study, no grouping of occytes was done before maturation of occytes with respect to presence or absence of cumulus cells. Further, Enright *et al.* (2000) investigated the effect of duration of maturation culture (16～32 hours) on the developmental competence of the oocyte. For optimal quality, it was concluded that maturation should be carried out for 24 hours (Enright *et al.*, 2000). By consideration of these facts, in the subsequent experiment, 24 hours duration was used for maturation culture of oocytes.

In the present study, protein supplementation in culture medium influenced the *in vitro* maturation rate of oocytes as indicated by presence of significant difference in maturation rate between oocytes cultured in medium supplemented with serum (FBS) or BSA. As protein supplementation in culture medium for *in vitro* maturation of oocytes, usually foetal bovine serum (FBS) or foetal calf serum (FCS) or oestrus cow serum (OCS) or BSA is used. Accordingly, Holm *et al.* (1999) stated that BSA is essential during *in vitro* maturation of bovine oocytes. Moreover, BSA was used with culture media as a source of protein by Rose and Bavister (1992). However, Sanbuissho and Threlfall (1988) found that FCS was superior to BSA as a protein supplement in maturation medium. The higher rate of maturation in FBS than BSA supplemented medium may be due to stimulation of oocytes maturation by different hormones present in serum. However, serum may act as a source of disease transmission in culture medium. Nevertheless, it has been documented that BSA may probably be chemically impure and contaminated with some low molecular weight compound (Kane, 1987). On the other hand, FBS has also been demonstrated as important protein supplement in oocytes maturation medium (Kobayeshi *et al.*, 1994). Alm *et al.* (2008) used 20% FBS in maturation medium and Das *et al.* (2006) used 15% FBS in maturation medium. However in the present study, 10% FBS was used in maturation medium for supplementation as protein source which was similar to other studies (Bhuiyan *et al.*, 2004; Talukder *et al.*, 2008).

In conclusions, both culture in droplets and 4-well dish are equally effective for *in vitro* maturation of oocytes of zebu cows. The COCs of zebu cows may be cultured for 18 to 27 hours for *in vitro* maturation without compromising the rate of maturation. Culture medium for *in vitro* maturation of zebu cows should be supplemented with FBS as protein source. Further studies are needed with more number of oocytes to confirm the finding as few numbers of oocytes were used in this study.

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