



***In vitro* production of bovine blastocyst with oocyte collected from abattoir ovary**

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Abstract

This study was designed to adopt *in vitro* embryo production (IVP) protocol using abattoir ovary. Ovaries were collected from local abattoir; cumulus-oocyte-complexes (COCs) were aspirated from 3 to 8 mm diameter follicles using a 10 ml disposable syringe attached with a 21G needle. The COCs were selected based on morphological characteristics and selected COCs were transferred into *in vitro* maturation (IVM) medium for 22 to 24 hrs. The matured COC were fertilized *in vitro* (IVF) using fresh semen capacitated through incubation with heparin sodium salt (20 µg/ml). After IVF, the presumed denuded zygotes were cultured in *in vitro* culture medium-I (IVC-I) for 3 days. After 3 days, the ≥8 cell embryos were transferred into IVC-II medium until day 8 of embryonic development (day 0: day of IVF). Cleavage and blastocyst development rates were evaluated at day 3 and day 7. The maturation rates of COC were examined through detection of first polar body and cumulus cell expansion. Results showed that 74.16 ± 5.49% of the total immature COCs were matured as detected by the presence of first polar body. The diameter of matured COC was 2.21 times higher than that of the immature COC. Moreover, about 64.30 ± 6.71% COC showed full expansion of their cumulus cell. The cleavage rate of presumed zygotes was 62.05 ± 7.07%. Among the cleaved embryos, 26.67 ± 11.78%, 10.84 ± 6.13%, 22.51 ± 9.57% and 39.98 ± 5.25% embryos were at 2-, 4-, 8- and 16 to 32-cell embryonic stages, respectively at day 3. Blastocyst development rates were 14.95 ± 4.39%. This study inferred that the BLRI adopted culture system supports development of bovine embryo *in vitro*.

Key words: Bovine, *in vitro* maturation, cumulus cell expansion, cleavage, blastocyst

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Introduction

Cattle of Bangladesh are mostly indigenous type and about 35% of indigenous cattle were crossed with exotic high yielding dairy and beef breeds (DLS, 2013). Production performances of the cattle population are generally low though they possess higher genetic biodiversity and popularly known for their higher reproductive efficiency. However, a very small number of cows are yielding 2 to 3 times higher milk than their population average (Deb *et al.*, 2012). In recent years, farmers are interested to raise high yielding cows rather than rearing number of low yielding cows. Therefore, rapid multiplication and distribution of existing available high yielding cows can increase milk production of the country within shortly. *In vitro* embryo production (IVP) and embryo transfer techniques are using in many developed and developing countries for rapid multiplication of high yielding cows.

The IVP technique accelerate genetic gain through increasing selection intensity and reducing generation interval (Camargo *et al.*, 2005; Marquez and Garrick, 2007; Manjunatha *et al.*, 2008). BLRI has undertaken research program to adopt IVP technology for multiplication of high yielding cows of Bangladesh since 2011. A consistent embryo culture, storage and transfer system to recipient cows is the pre-requisite for application of IVP technology with the traditional cattle breeding program. The ovary of slaughtered female cattle is a cheap source of oocyte for research purposes and is commonly used for standardization of *in vitro* embryo production, embryo cryopreservation and embryo transfer to recipient heifer/cows protocols (Deb *et al.*, 2011; Dey *et al.*, 2011; Ha *et al.*, 2013a and 2013b). Considering these facts, this study was designed to establish *in vitro* embryo production system using oocyte from abattoir cow ovaries.

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Materials and methods

In vitro maturation

Ovaries of slaughtered cows were collected from abattoir located at Mohammadpur, Dhanmondi, Dhaka in physiological saline (0.90% sodium chloride supplemented with 100.00 IU/ml penicillin and 0.10 g/ml streptomycin sulfate) at ambient temperature and transported to the laboratory within 4 to 5 hrs of slaughter. In the laboratory, extraneous tissue was removed and ovaries were washed with phosphate buffer saline (PBS). The cumulus-oocyte-complexes (COC) were aspirated using a 10 ml disposable syringe attached with a 21G needle. The aspirated material was poured onto a 100.00 mm Petridis containing TL-HEPES (114.00 mm sodium chloride, 3.20 mm potassium chloride, 2.00 mm sodium bicarbonate, 0.34 mm sodium biphosphate, 10.00 mm sodium lactate, 0.50 mm magnesium chloride, 2.00 mm calcium chloride, 10.0 mm HEPES, 1.00 µl/ml phenol red, 100.00 IU/ml penicillin, and 0.10 mg/ml streptomycin) solution and the cumulus-oocyte-complexes (COC) were searched under a microscope at low magnification (4x).

The COCs possessing an even cytoplasm and covered with minimum 3 layers of compact cumulus cells were selected for *in vitro* maturation (Stojkovic *et al.*, 2001). The selected COC (50 to 70 per well) were washed 2-3 times in TL-HEPES and 2-3 times in IVM medium (TCM199 + 10.00% FBS, 1.00 µg/ml β-estradiol, 10.00 µg/ml FSH, 0.60 mm cystein, and 0.20 mm sodium pyruvate) before placing them into a well of 4-well dish containing 500.00 to 700.00 µl IVM medium for 22 to 24 hrs.

In vitro fertilization

The matured COCs were fertilized *in vitro* by fresh semen collected from BLRI cattle Breed-1 (BCB-1) using artificial vagina method. Hundred micro litters (8.00 to 10.00 µl) semen were placed in a 15 ml conical tube containing 10.00 ml D-PBS and pelleted by centrifugation at 750×g for 5 min. The supernatant was removed carefully and 10.00 ml D-PBS was added in the tube. The sperm was washed for 2 to 3 times accordingly. Then sperm were capacitated through incubation with 500.00 µl IVF medium (Tyrode's lactate

solution supplemented with 6.00 mg/ml BSA, 22.00 µg/ml sodium pyruvate, 100.00 IU/ml penicillin, and 0.10 mg/ml streptomycin) containing heparin sodium salt (20.00 µg/ml) for 15 min. After capacitation, spermatozoa were diluted at approximately 1×10^6 spermatozoa/ml with IVF medium. The matured COC were cocultured with capacitated spermatozoa for 18 to 20 hrs through placing them into a well of 4-well dish (500.00 to 700.00 µl).

In vitro culture

After IVF, the cumulus cells were removed by gentle pipetting into TL-HEPES and the denuded presumed zygotes were placed in the well of a four-well dish containing 500.00-700.00 µl CR1-aa medium (Rosenkrans *et al.*, 1993) supplemented with 44.00 µg/ml Na-pyruvate, 14.60 µg/ml glutamine, 10.00 µl/ml penicillin/streptomycin, 3.00 mg/ml BSA and 310.00 µg/ml glutathione for 3 days (IVC-I). These were then cultured until day 8 of embryonic development in a medium of the same composition, except that the BSA was replaced with 10.00% (v/v) FBS (IVC-II). The incubation conditions during IVM, IVF and IVC were 5% CO₂ in air at 38.5°C with maximum humidity. The media (IVM, IVF and IVC) were pre-incubated for minimum two hours under culture condition.

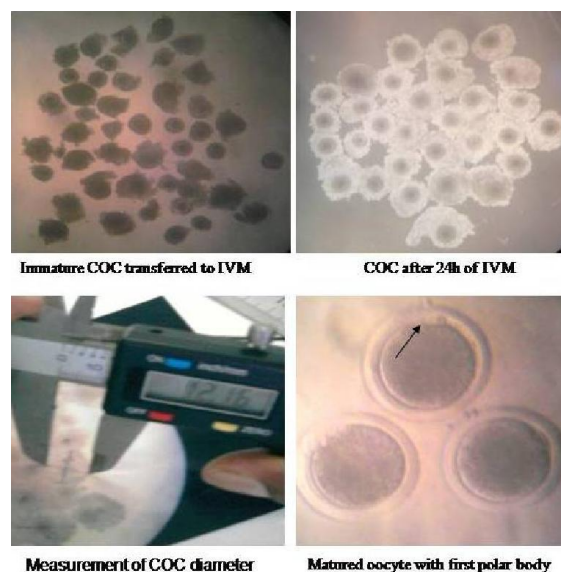


Figure 1. Detection of maturation rates after *in vitro* maturation of bovine cumulus-oocyte-complexes. Arrow head indicates presence of first polar body

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Efficiency of culture system

The efficiency of the studied culture system was measured by evaluating i) maturation, ii) cleavage and iii) blastocyst development rates. The maturation rates of COC were examined through detection of first polar body under stereomicroscope (Dey *et al.*, 2011) and cumulus cell expansion (Rahman *et al.*, 2003). After maturation, the COCs were denuded and first polar body was checked under stereomicroscope. Measure on cumulus cell expansion was conducted according to Rahman *et al.* (2003). The COCs were graded into three groups according to cumulus cell expansion i.e. full (FE), medium (ME) and less (LE) expansions. Moreover, the diameters of COC at onset and after IVM were measured using slide calipers (Figure 1). The cleavage rate was calculated based on the number of presumed zygote transferred to IVC-I medium and number of the cleaved to at least 2-cell embryonic stage. Similarly blastocyst development rates were calculated at day 8 on the basis of total blastocyst counted and presumed zygote transferred into the IVC-I medium. The data were represented in percentage.

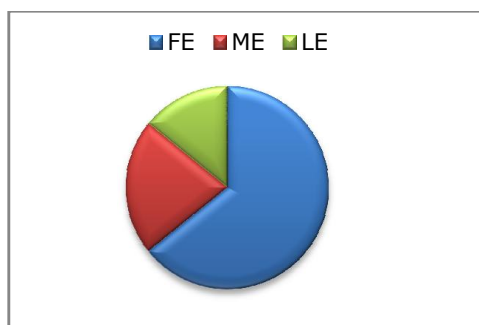


Figure 3. Cumulus cell expansion (%) in bovine cumulus-oocyte-complexes matured *in vitro*. FE: full expansion, ME: Medium expansion and LE: least expansion

Statistical analysis

Eight replications for estimation of embryonic development rate (cleavage, 2-, 4-, 8- and ≥ 16 -cell embryonic stage) and that of three for blastocyst development rate were used. Mean and standard deviation was calculated using MS Excel.

Results and Discussion

The maturation rate following 22 to 24 hrs culture in IVM medium was $74.16 \pm 5.49\%$ (142 out of 192) as detected by extrusion of first polar body (Figure 1). The diameter of immature and matured COCs was 6.19 ± 0.91 and 13.66 ± 2.38 mm, respectively. The diameter of COC was increased 2.21 times following maturation *in vitro*. About $64.30 \pm 6.71\%$ COCs showed full expansion of their cumulus cell during IVM (Figure 2). The cleavage rate was $62.05 \pm 7.07\%$. Among the cleaved embryos, $26.67 \pm 11.78\%$, $10.84 \pm 6.13\%$, $22.51 \pm 9.57\%$ and $39.98 \pm 5.25\%$ were at 2-, 4-, 8- and 16 to 32-cell embryonic stages, respectively (Figure 3 and 4). Moreover, blastocysts development rate was $14.95 \pm 4.39\%$ (Figure 4).

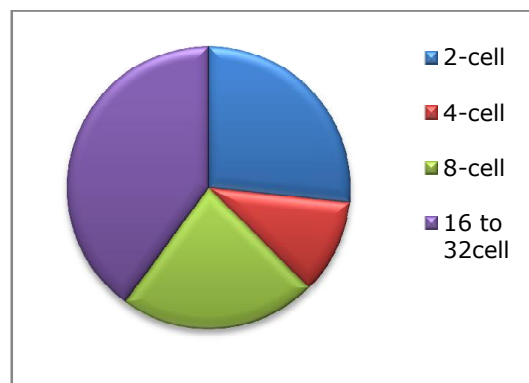


Figure 2. Development rates (%) of bovine embryos at different embryonic stages observed at day 3 (day 0: day of *in vitro* fertilization)

Many developed and developing countries have been using IVP technique for increasing production performance of their cattle population since last couple of years. However, the IVP technology is still at research level in Bangladesh. BLRI has been conducting researches on IVP since 2011 to apply this technique with traditional cattle breeding system in Bangladesh. To our best knowledge, this is first time for Bangladeshi scientist to produce bovine blastocyst using COCs from slaughtered cows. The maturation, cleavage and blastocyst development rates of BLRI adopted IVP were $74.16 \pm 5.49\%$, $62.05 \pm 7.07\%$ and $14.95 \pm 4.39\%$, respectively.

Maturation rates of COCs found in the current study are within the range of normal standard culture system. During IVM, 60.00 to 85.00% of the immature oocyte normally develops to metaphase II stage (Dey et al., 2011). Nuclear maturation of *in vitro* matured bovine oocyte is commonly evaluated through detection of first polar body in the perivitelline region of the oocyte (Dey et al., 2011). Immature oocytes are generally arrested at diplotene stage and resume their meiosis during maturation period. Resumption of meiosis is essential for preparation of the oocyte for fertilization. The first polar body is extruded during meiosis process and it indicates completion of the first meiotic division in oocytes. Diameter and cumulus cell expansion of COC, associated with maturation of oocytes were also evaluated for monitoring maturation rates of COC. As maturation progress, the diameter of COC is increased. Degree of cumulus cell expansion is associated with *in vitro* oocyte maturation rate (Rahman et al., 2003; Jahan et al., 2012). This study also revealed that cumulus cell expansion is also associated with maturation status of oocyte. Collectively, these results showed that the BLRI adopted IVP is enable to support normal maturation of bovine COC *in vitro*.

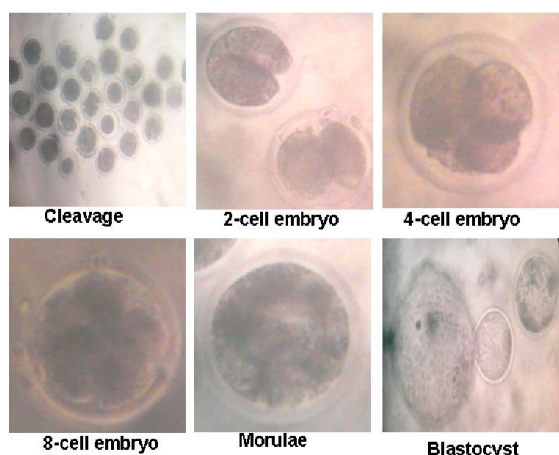


Figure 4. Bovine *in vitro* produced embryos at different developmental stages

Development rates to cleavage and blastocyst was lower in the current study than standard culture system (Dey et al., 2011). However, some reports showed similar and/or lower cleavage rates compared to present study (Livingston et al., 2014). Among the cleaved

embryos, $26.67 \pm 11.78\%$, $10.84 \pm 6.13\%$, $22.51 \pm 9.57\%$ and $39.98 \pm 5.25\%$ were at 2-, 4-, 8- and 16 to 32- cell embryonic stages, respectively. The efficiency of *in vitro* embryo development depends on oocyte donor, semen quality, and culture media and culture conditions employed during each phase of IVP (Brum et al., 2005). Beside these factors, the IVP efficiency is also affected by osmolality of media (Gordon, 1994), water quality used in different steps of IVP (Fukuda et al., 1987) and gasing level of incubator (Wang, 1991) among others. In this study, osmolality of different media was not checked due to lack of equipment facility. The CO₂ level was recorded through monitoring display system of the incubator. The actual internal CO₂ level of incubator was not confirmed through using externally measurable devices. In some cases, the display system of incubator may not show actual internal condition. Therefore, the low embryonic development of this study might be associated with incubation condition (CO₂ level and temperature), media osmolality and other uncontrolled factors.

Conclusion

The present culture system supports normal *in vitro* maturation of immature oocytes and subsequent embryonic development to blastocyst stage. Therefore, this culture system may be used with traditional cattle genetic improvement programme of the country.

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