~~Establishment~~ Development of bovine embryo culture system for maintaining genetic identity of embryos

ABSTRACT

Cumulus-oocyte complexes (COCs) recovered during an ovum pick-up (OPU) session from a cow is low (1 to 15 COCs). Moreover, mixing of two or more cows COCs to increase total number in a culture droplet will loss the genetic identity of embryos. ~~Considering above facts,~~ The present study was aimed to develop a culture system suitable for culture of smaller number of COCs (less than 10 COCs). Slaughter house bovine ovaries were used ~~during this study.~~ Aspirated oocytes possessing an even cytoplasm and covered with minimum 3 layers of compact cumulus cell were subjected to *in vitro* maturation (IVM) followed by *in vitro* fertilization (IVF) using fresh semen capacitated by treating with heparin sodium salt. Cumulus cells were removed by gentle pipetting and denuded zygotes were subjected to *in vitro* culture (IVC) for blastocyst development. Cleavage rates were evaluated at day 3 (day 0: day of IVF) and blastocyst rate at day 8 as a proportion of the presumed zygote transferred into IVC-I medium. Results show that development rate up to cleavage did not affect by COCs densities. Single COCs did not reach ~~to~~ the blastocyst stage when cultured individually. The development rate to the blastocyst stage was 13.9±9.0%, 10.8±4.9% and 16.6±3.9% for 3, 5 and 10 embryo groups, respectively. ~~Alternatively,~~ The GPS group culture allowed the smaller numbers of COCs (1, 2, 3 and 4 COC per chamber) to develop at blastocyst stage. ~~In conclusion,~~ The present experiment developed *vitro* embryo production system for small number of bovine oocytes.

Keywords: Bovine, cumulus-oocyte-complexes, cleavage**,** blastocyst, drop culture

1. INTRODUCTION

Bangladesh possesses large number of cattle population composing of indigenous, their crosses with different exotic breeds and few pure exotic breeds. About 24.856 million cattle make up Bangladesh's bovine population, of these, 6 million are dairy cattle, 85 to 93.3 percent are indigenous Zebu type (Bos indicus) cattle, and the remaining 6.7 to 15 percent are crossbreds (Hamid *et al.,* 2017, Hossen *et al.,* 2008, DLS 2022). Adult indigenous cattle have body weight of 150 -250 kg and produces between 47 and 52 percent meat by weight with a very lower milk yield (Samad, 2020). However, there are some cows in the available population that are yielding two or three times more than the average for their population. Application of reproductive biotechnology tools to multiply these high yielding cows can enhance dairy/meat production of the country through increasing number of high yielding cows. *In vitro* embryo production technology in conjunction with ovum pick and embryo transfer techniques are routinely being used for multiplication of high yielding cows in many developed and developing countries (Kong *et al.*, 2011).

The efficient use of immature oocytes in follicles has been made possible by the widespread development and modification of in vitro maturation (IVM) methods that use mammalian oocytes (A'Arabi et al., 1997, Hughes et al., 2010). Numerous industries, such as the animal husbandry business, the conservation of wild animals, and assisted human reproductive technologies, depend on IVM technology. To evaluate the effectiveness of in vivo embryo

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development after in vitro fertilization and in vitro culture (IVF–IVC), extensive research has been conducted. But as of right now, in vitro-matured oocytes are less fertile and have poorer developmental competence than in vivo-matured oocytes (Eppig et al., 1989). So it is crucial to enhance IVM conditions to enhance the in vitro embryo production efficiency. The number of cumulus-oocyte complexes (COCs) in IVM affects the oocyte maturation rate, since the paracrine and autocrine factors that are necessary for oocyte development are maintained by the cumulus cells and oocytes (Eppig et al., 2001). High paracrine and autocrine activity are reportedly attained in cultures containing more than 20 COCs, according to the findings of a previous study (Eppig et al., 2001).

The ultrasound-guided transvaginal ovum pick-up (OPU) based *in vitro* embryo production (OPU-IVP) facilitates multiplication of high yielding cows without altering their genetic make-up (Ward *et al.,* 2000). The technique is applicable irrespective of physiological condition of donor including i) early pregnancy, ii) stage of reproductive cycle, iii) donor age- 6 months age and onwards (Reis *et al.,* 2002; De Roover *et al.,* 2008; Merton *et al.,*2009). Therefore, the number of high yielding dairy cow can be increased within shortest possible time frame through application of OPU-IVP. However, lower recovery (1 to 15 COCs) of cumulus-oocyte complexes (COCs) form individual donor during OPU session results poor embryo production efficiency. Moreover, mixed culture of two or more cows COCs in a culture droplet will loss the genetic identity of embryos. Considering the above facts, the present study was aimed to develop a culture system suitable for culture of smaller number of COCs (less than 10 COCs).

2. MATERIALS AND METHODS

2.1 *In vitro* embryo production

A 10-mL disposable syringe fitted with a 21G needle was used to extract cumulus-oocyte-complexes (COCs) from 3 to 8 mm diameter follicles of slaughtered cow ovaries. The COCs that had at least three layers of dense cumulus cells covering them and an even cytoplasm were chosen for in vitro maturation (IVM). The chosen COCs were, in accordance with the experimental design, washed twice in TL-HEPES and then twice in IVM medium before being placed in an IVM droplet. After being dissolved in the IVF medium for 15 minutes, fresh semen was used to facilitate in vitro fertilization (IVF) of the matured COCs. The IVF medium was used to dilute the capacitated sperm at a rate of roughly 1×10^6 spermatozoa/mL. For eighteen to twenty hours, capacitated spermatozoa were co-cultured with matured COCs. Cumulus cells were extracted from IVF-treated TL-HEPES by gently pipetting. After being rinsed three times in in vitro culture (IVC) medium-I, the denuded zygotes were incubated for three days in the culture droplet. Following a three-day incubation period, the 8 to 32 cell embryos were moved onto ICV-II media (until Day 8). At 38.5°C and maximum humidity, the incubation conditions for IVM, IVF, and IVC were 5% CO2 in air. As a percentage of the assumed zygote injected into IVC-I medium, cleavage development rates were assessed on day three (day 0: the day of IVF). Day 8 blastocyst development rates were computed as a percentage of the assumed zygote placed in IVC-I medium.

2.2 Experimental design

This experiment was conducted to standardize IVP protocol for small number of oocytes. For this purpose, two culture systems including conventional droplet culture on 35-mm Petridis and GPS group culture were tested using different oocyte densities at IVM. In conventional droplet, COCs were cultured in 4 different densities- i) Group A: 1 COCs per droplet, ii) Group B: 3 COCs per droplet, iii) Group C: 5 COCs per droplet and iv) Group D: 10 COCs per 120 µL droplet medium covered with mineral oil. For GPS group culture (GPH), total 10 COCs were placed under a 120 µL droplet. The ten COC were divided into four group (GPH-

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1:1 COCs per chamber, GPH-2:2 COCs per chamber, GPH-3:3 COCs per chamber and GPH-4:4 COCs per chamber) and placed into four chamber under a 120-uL droplet. The fence of a chamber was made by vertical posts so that the COCs of a chamber cannot move but the culture media can move from one chamber to another. All of these experiments were replicated six times.

2.3 Data analysis

Data were expressed as mean values with standard deviation (Mean±SD) and one way ANOVA followed by Tukey post-hoc test was conducted by SPSS statistical package (version 25.0). Statistically significance was considered when $p<0.05$.

3. RESULTS AND DISCUSSION

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Table 1: *In vitro* embryo development efficiency in conventional droplet culture system

Group	% Cleaved (Mean±SD)	% Blastocyst (Mean±SD)
Group A (1 COCs)	50.0±22.4	$0.0^{b}\pm0.0$
Group B (3 COCs)	72.2±10.2	$13.9^{a}\pm9.0$
Group C (5 COCs)	71.7±3.8	$10.8^{a}\pm4.9$
Group D (10 COCs)	75.5±2.7	$16.6^{a}\pm3.9$

$^{a–b}$ Within a column, means without common superscripts differ (P<0.008)

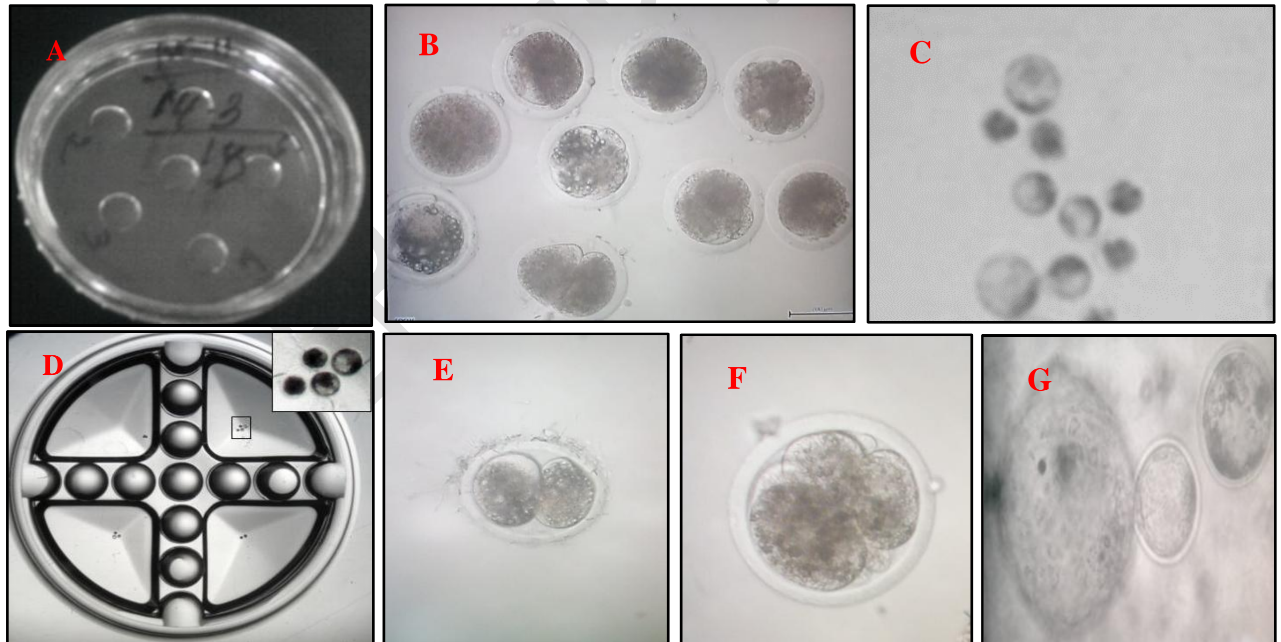

Fig1. COCs developmental competence at droplet and GPS culture A) COCs cultured in droplet culture; B) 2-cell, 4-cell and morula stage at droplet culture; C) Blastocyst at droplet culture system; D) COCs cultured in GPS; E) 2-cell at GPS F) 4-cell at GPS; G) Developed blastocyst at GPS

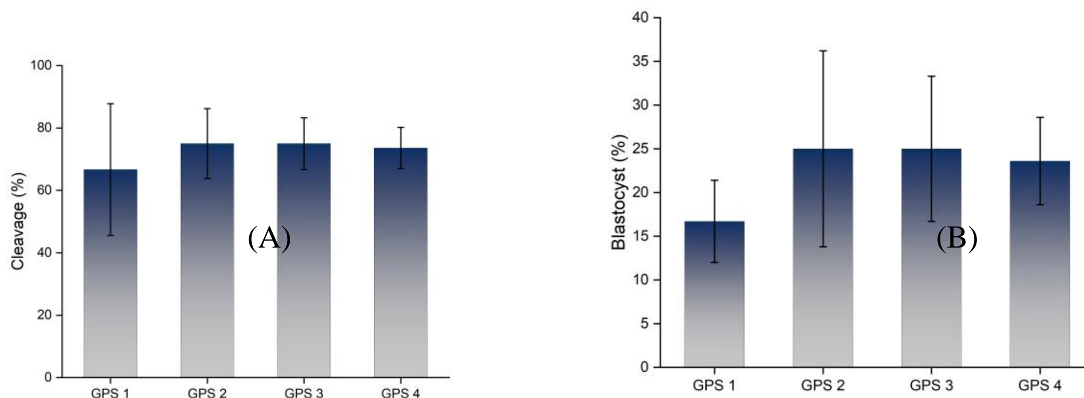

Fig 2.*In vitro* embryo development efficiency in GPS culture system (A-Cleavage%, B-Blastocyst %) (GPS 1:1 COCs, GPS 2:2 COCs, GPS 3:3 COCs, GPS 4:4 COCs)

~~This experiment was conducted to standardize *in vitro* embryo production protocol for small number of oocytes.~~ Remove Result shows that development rate up to cleavage did not affect ~~by~~ the COCs densities (Table 1). Single COCs did not reach ~~to~~ remove the blastocyst stage when cultured individually in droplet culture. The development rate ~~to~~ at the blastocyst stage was 13.9±9.0%, 10.8±4.9% and 16.6±3.9% for groups of 3, 5, and 10 embryos respectively (Table 1). Alternatively, the GPS group culture allowed the smaller numbers of COCs (1, 2, 3 and 4 COC per chamber) to develop at blastocyst stage (Figure 2). The cleavage and blastocyst rate of slaughter house driven COCs in conventional culture dish was found to be 75.5±2.7% and 16.6±3.9% which are in accordance with our current GPS and drop culture cleavage and blastocyst rate (Akter *et al.,* 2020). Different plastic dishes with varying sizes and shapes are used to culture gametes and COCs. The experiment's goal and the laboratory's preferences play a major role in the selection of this culture system. Nonetheless, studies reveal that through secreting trophic autocrine/paracrine substances, higher embryo densities enhance the developmental capacity of the embryo (Swain *et al.,* 2011). Indeed, a number of substances that influence embryo developmental competence are secreted by developing embryos (Bormann *et al.,* 2006; Katz-Jaffe *et al.,* 2006). For this reason, it is believed that Petri dishes with a confined surface area that use a lesser volume of media are better for developing embryos than dishes with a larger surface area (Swain *et al.,* 2011).

Cattle *in vitro* embryo production is directly affected by the number of oocytes/embryos cultured. Compared to grouped IVM oocytes, singly cultured oocytes exhibited lower levels of maturity and developmental competence (Nishio et al., 2014). Less than 20 oocytes/embryos per group results poorer embryo production rates than cultures in groups with more number of oocytes (Donnay et al., 1997; O'Doherty et al., 1997). A recent study suggest that, the development of blastocysts is less when five oocytes are matured in vitro in a group than when 10 or 20 COCs are. Nevertheless, cleavage and blastocyst growth rates are unaffected by the amount of medium (1 vs. 5 vs. 10 µl per COC) employed in vitro maturation (Brum et al., 2005). Single COCs cultured in drop culture under our experimental conditions cannot develop past the cleavage; nevertheless, when single COCs are cultured in GPS dishes, they can develop into blastocysts. This is most likely because growing embryos and COCs release autocrine and paracrine substances that have a stimulatory impact (O'Doherty et al., 1997).

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Oocyte/embryo secreted factors mediate the autocrine and paracrine actions. Cattle and other species' embryonic development is regulated by these impacts. The bovine embryonic genome is activated at the 8-cell stage, and during the maturation period, oocytes carry the chemicals needed from their mother to maintain embryonic development until the 8-cell stage. Therefore, according to O'Doherty et al. (1997) and Brum et al. (2005), the rate of embryonic growth up to the 8-cell stage is typically unaffected by the culture system, embryo densities, and autocrine and paracrine influences. The embryo's own genome is activated after the 8-cell stage, and further development is dependent on a number of variables, such as culture medium, incubation conditions, embryo densities, culture devices, and others.

Traditional Petri dishes, which are flat and sterile, are the most often used platform for embryo culture. Newer dishes specifically made for embryo culture are available from a number of vendors. The idea behind the recent dishes is to incorporate conical, smaller-volume wells into the dish design, allowing embryos to rest where the wells are lowest and potential sites for the concentration of embryotrophic substances (Reed, 2012). GPS cultures dishes are small round-bottom wells added to a standard Petri dish modification allow cultivated embryos to be closely clustered together. Such dishes help with cell recovery and identification and can prevent micro drop issues such as aggregation or moving of drops. This GPS dish allows for the sharing of a media reservoir and also makes it easier to observe embryos because one embryo may be cultured in each partition (Azouz et al., 2021). It can take some time for the embryos in the bottom of the concave microwell to settle centrally because of gravity and that may facilitate the development of fewer numbers of COCs to embryo in GPS culture dish.

4. Conclusion

Considerable rate of blastocyst ~~is~~ was achieved through GPS culture system in comparison with drop culture for small number of COCs. Since ovum pick up resulted to very low number of COCs and ~~this~~ these COCs can be processed without compromising their developmental competence under GPS culture system.

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