International Journal of Livestock Research, Vol. 13 (5) May' 2023
http://www.ijlr.org
eISSN: 2277-1964

Original Research

Effect of Single Vs. Group Culture System On *In Vitro* Maturation and Embryo Development in Bovine

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How to cite this paper: Khatun, H., & Islam, M. R. (2023). Effect of Single Vs. Group Culture System On In Vitro Maturation and Embryo Development in Bovine. *International Journal of Livestock Research*, 13 (5), 8-15.

Received : Apr 13, 2023
Accepted : May 18, 2023
Published : May 31, 2023

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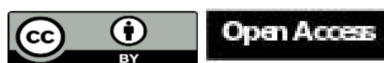

Abstract

The first objective of this research was to compare the effect of single vs. group culture methods on the developmental rate of oocyte maturation and embryo production in vitro. Next, to investigate the quality of blastocyst derived from single vs. group culture methods. Bovine cumulus-oocyte complexes (COCs) derived from slaughterhouse ovaries were matured in single (1 COCs/drop) and group (20 COCs/drop) methods for 22 h followed by 6 h in vitro fertilization, and zygotes were cultured for 8 days. To evaluate the blastocyst quality TUNEL Assay was performed on day 8 of IVC. The results showed that single culture methods significantly decreased the rate of oocyte nuclear maturation (90.1 ± 1.5 vs. 81.2 ± 2.5; P<0.01) and subsequent embryonic development (47.6 ±2.2 vs. 41.5 ±1.9; P < 0.05) compared to the group culture methods. In contrast, the rate of fertilization and the number of total cells in blastocysts were similar between single vs. group culture methods. In addition, there was a tendency to decrease the percentage of apoptosis cells in blastocysts derived from a single method than that observed in the group culture method. In conclusion, the group culture method had higher developmental competence of oocytes compared to the single method; however, in terms of blastocyst quality, no significant difference was found between the two groups of culture methods. These findings suggest that improvement of single culture methods will be the best option to reduce the constraints associated with group culture methods and may be contributed to quality embryo transfer in either animals or humans.

Keywords: Blastocysts Quality, Bovine Oocyte, Group Culture, *In vitro* Maturation, *In vitro* Embryo Culture, Single Culture.

Introduction

In worldwide, the methods of *in vitro* production (IVP) of embryo have advanced day by day. The bovine IVP embryo model has been considered as an excellent model for studying infertility and treatment in humans. Despite numerous advances in livestock IVP protocols, there is ample evidence showing that still exist great differences between *in vivo* and *in vitro* produced embryos in both morphological and molecular aspects (Lonergan *et al*., 2006; Lonergan *et al*., 2001; Paramio & Izquierdo, 2016; Suzuki *et al*., 2000). *In vitro* culture conditions have been considered as a critical issue that can compromise many aspects of oocyte and embryo development (Huang *et al*., 2015; Mauchart *et al*., 2023; Xu & Qiao, 2021). Moreover, the number of oocyte or embryo cultures in a group is also associated with quality and developmental competence. Because, the oocyte comprises cumulus cells and oocyte, and they maintain the supply of paracrine and autocrine factors during *in vitro* maturation (IVM) which are required for oocyte developmental acquisition (Gilchrist & Thompson, 2007; Hao *et al*., 2022; O'Neill, 1997). It has been reported that high paracrine and autocrine activities have occurred when more than 20 COCs were cultured in a group (Guo *et al*., 2018; Su *et al*., 2003; Su *et al*., 2002; Zhang *et al*., 2010). However, grouped oocyte or embryo culture has many drawbacks for use in the animal industry and experimental animal research studies. For example- grouped IVM oocytes are indistinguishable from each other, and therefore, it is very difficult to differentiate them. In addition, it requires the use of many animals for one experiment. Similarly, in group culture, different embryos may differentially secrete or utilize substances into/from the culture medium. Thereby, some factors derived from poor-quality embryos may have negative influences on the development of the surrounding embryo (Hoelker *et al*., 2010; Tao *et al*., 2013; Tao & Liu, 2013; Vajta *et al*., 2008). A previous study reported that group culture of embryos prior to Day 2 or Day 3 transfer in humans has no positive value (Shi *et al*., 2022; Tao *et al*., 2013). Furthermore, in some cases, the group culture of embryos to the blastocyst stage is not always practical because not all patients are blastocyst culture candidates. Therefore, culturing oocytes/embryos separately according to their quality is useful for selection purposes.

Therefore, the aim of the present study was to investigate the effect of single vs. group culture methods on the developmental rate of oocyte maturation and embryo production *in vitro*. Next, to investigate the quality of blastocyst derived from single vs. group culture methods.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma unless otherwise indicated (St. Louis, MO, USA).

Oocyte Collection and IVM

Bovine ovaries were collected from a local abattoir and washed at least three times in a sterile saline solution. Then cumulus-oocyte complexes (COCs) were aspirated from follicles (2–6 mm in diameter) using a 19-gauge needle attached to a 10-mL syringe. COCs were washed three times with TCM-199 (Thermo Fisher Scientific) containing 5% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific) followed by maturation in IVM medium TCM-199 supplemented with 5% FBS, follicle-stimulating hormone (0.02 IU/mL; Kyoritsu Seiyaku, Tokyo, Japan), and gentamicin (10 µg/mL, Nacalai Tesque, Kyoto, Japan) covered with liquid paraffin (Nacalai Tesque) at 38.5°C for 22 h in a humidified atmosphere of 5% CO_2 in air.

In vitro Fertilization

In vitro fertilization (IVF) was carried out according to a previously described procedure (Khatun *et al*., 2020). Briefly, frozen semen was thawed by immersing the straw in warm water (37°C) for 20 s. Spermatozoa were washed by centrifugation (800 g for 10 min) in 90% (v/v) percoll solution (GE Healthcare). After removing the supernatant, the pellet was diluted with IVF100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) and centrifuged at 600 g for 5 min. The spermatozoa pellet was then diluted with IVF100 to prepare a final sperm-cell concentration of 5.0×10^6 sperm/mL. Following IVM, COCs were washed three times with IVF100 and placed into an IVF100 sperm drop covered with liquid paraffin. IVF was performed at 38.5°C in 5% CO2 in the air under humidified conditions for 6 h.

in vitro Embryo Production

After IVF, cumulus cells were removed mechanically by pipetting in CR1aa medium containing 5% FBS, and putative zygotes with polar bodies were placed into drops of CR1aa medium supplemented with 5% (v/v) FBS. The drops were then covered with liquid paraffin and *in vitro* culture (IVC) carried out at 38.5°C in a humidified atmosphere of 5% O_2 and 5% CO_2 and balanced with N_2 through Day 8 (Day 0 represented the day of insemination).

Culture Conditions and Experimental Design

This study had two experimental treatments: single culture (1 COCs/drop) and group culture (20 COCs/ drop). After aspiration of COCs from bovine ovaries, COCs were subjected to IVM, IVF, and IVC by using single vs. group culture methods (Fig. 1). The volume of culture medium 100-µL droplets (group culture) and 20-µL droplets (single *culture*) were placed on the bottom of the 35-mm dishes.

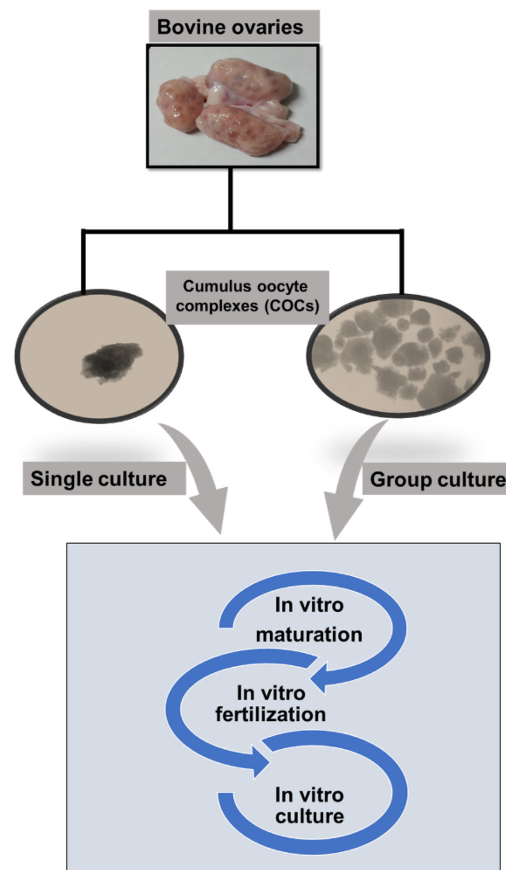

Figure 1 Schematic diagram of the culture protocol.

Assessment of Meiotic Maturation

After 22 h of IVM, meiotic maturation was assessed by acetic-orcein staining, based on nuclear stage of oocytes according to the procedures mentioned previously (Khatun *et al*., 2020). Briefly, oocytes were denuded by pipetting in IVM medium containing 0.1% hyaluronidase, washed with 0.1% polyvinylpyrrolidone (PVP; Nacalai Tesque) in Dulbecco's PBS (DPBS, Sigma). In brief, oocytes were denuded by pipetting in TCM-199 medium containing 0.1% hyaluronidase, washed with Dulbecco's PBS (DPBS, Sigma) containing 0.5 mg/mL polyvinylpyrrolidone (PVP; Nacalai Tesque) and mounted on microscope slides. The samples were fixed for 2–3 days with 25% (v/v) acetic acid in ethanol and stained with 1% acetic orcein (w/v) in 45% (v/v) acetic acid for 60 min. Meiotic stages of the oocytes were evaluated under a microscope (Eclipse Ti, Nikon).

TUNEL *Staining*

A TUNEL assay kit (in situ cell death detection kit; Roche) was used to *evaluate* the presence of apoptotic cells in

matured COCs and blastocysts at Day 8 of culture. Briefly, COCs/blastocysts were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 40 min, rinsed three times with 0.05% (w/v) PVP-PBS, and then permeabilized with 0.05% (w/v) PVP-PBS containing 0.5% TritonX-100 for 20 min followed by three washes with 0.05% (w/v) PVP-PBS for 5 min. The fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 38.5°C. After incubation, COCs/blastocysts were washed three times with 0.05% (w/v) PVP-PBS for 5 min each followed by mounting onto glass slides using a mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI; Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA). The fluorescence of the fragmented DNA ends was detected under a fluorescence microscope (BZ-X800, KEYENCE).

Statistical Analysis

Each trial of the experiment run was accompanied by parallel *control* and the same cultural conditions. For each experiment, at least three independent replicates were performed unless specified otherwise. The results are presented as the mean ± standard error (S.E.M.). All data were analyzed using analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. All percentage data were arcsine transformed prior to statistical analysis. A value of $P < 0.05$ and $P < 0.01$ were considered to be statistically significant.

Results

Effect of Single Vs. Group Culture Methods on Oocyte Meiotic Maturation

At first, we examined the effect of single vs. group culture methods on the nuclear maturation of oocytes by acetic-orcein staining (Table I). The results showed that group culture methods significantly increased the percentage of oocytes that progressed to the metaphase II (MII)-stage as compared with single culture *methods* (85.1 ± 1.4 vs. 62.3 ± 2.5; $P < 0.01$). In contrast, the percentage of metaphase I (MI)-stage of oocytes was significantly increased in the single culture method than in the group culture method. However, no significant difference was found in the rates of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) stages of oocytes between the culture methods (Table 1). Consequently, the rate of cumulus cell expansion was significantly lower in oocytes derived from the single culture method compared to that observed in the group culture method (Fig. 2).

Table 1 Effects of single vs. group culture methods on nuclear maturation of oocyte in bovine

Culture Methods	Drop Volume (µl)	No. of oocytes examined	Nuclear maturation stages (%)			
			GV	GVBD	MI	MII
Single (1 COCs/drop)	20	161	3.0 ± 2.1	6.4 ± 2.5	28.3 ± 2.0[a]	62.3 ± 2.5[a]
Group (20 COCs/drop)	100	162	2.4 ± 0.7	3.3 ± 1.2	9.2 ± 0.9[b]	85.1 ± 1.4[b]

Data are mean ± S.E.M. from four replicates. a, bValues with different superscripts within the same column are significantly different (P < 0.01). COCs, cumulus oocyte complex; IVM, In vitro Maturation; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Effect of Single Vs. Group Culture Methods on Fertilization and Embryo Development

To examine whether the single vs. group culture methods affect fertilization and early embryonic development, IVF followed by IVC was performed by using MII oocytes. As shown in Table 2, data obtained in the single culture method were significantly lower than those in the group method ($P < 0.05$). The pronuclei formation rates and the rate of development to the two-cell stage embryo in single methods did not differ from that of the group methods; however, the rate of total blastocysts formation was significantly decreased *(13.8 ± 1.7 vs. 42.5 ± 1.4; P < 0.05)* by single culture method than that observed in group culture methods.

Slaughterhouse-derived bovine oocytes were cultured in two different methods single vs. group for *in vitro* maturation (22 h) followed by *in vitro* fertilization (6 h) and zygotes were cultured until Day 8. The rate of fertilization was determined by counting the number of zygotes containing two pronuclei. The developmental competence of embryos was examined on Days 2 and 8 of culture. Data are mean ± S.E.M. from five replicates. [a,]

[b] Values with different superscripts within the same column are significantly different ($P < 0.05$). IVM, *In vitro* Maturation, h, hour.

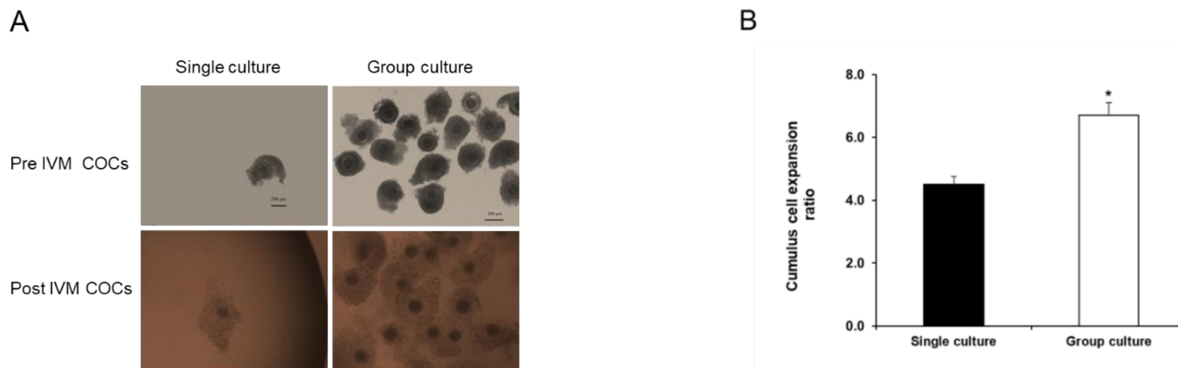

Figure 2: Effects of single vs. group culture methods during IVM on cumulus cell expansion. (A) Representative images of COCs at pre- and post-IVM. (B) The ratio of cumulus cell expansion of COCs obtained from of single vs. group culture methods. Scale bar: 200 µm. Values are means ± S.E.M. from three independent experiments. *: $P < 0.05$. Cumulus cell expansion ratio = mean of total COC area after maturation/mean of total COC area before maturation in each group. IVM, *in vitro* maturation; COCs, cumulus cell complexes.

Table 2: Effects of the single vs. group culture methods on oocyte fertilization and embryonic development in bovine

Culture methods	No. of MII oocyte cultured	Multiple-pronuclei (%)	Two-pronuclei (%)	≥ 2 cells embryos at day 2 (%)	Total Blastocysts at day 8 (%)
Single (1 COCs/ 20 µl drop)	110	12.6 ± 0.9	87.4 ± 1.6	88.2 ± 2.3	13.8 ± 1.7[a]
Group (20 COCs/100 µl drop)	120	9.2 ± 0.8	90.8 ± 0.8	91.7 ± 2.3	42.5 ± 1.4[b]

Evaluation of Blastocyst Quality Derived from Single Vs. Group Culture Methods

Next, the quality of blastocysts in each group was evaluated by Tunnel Assay (Fig. 3). The total cell number of blastocysts between the groups did not significantly differ but the percentage of tunnel-positive cells has a tendency to decrease in oocytes derived from single culture method. These results suggest that although the numbers of blastocysts obtained by the single culture method were decreased, the quality of blastocysts did not differ between the culture methods.

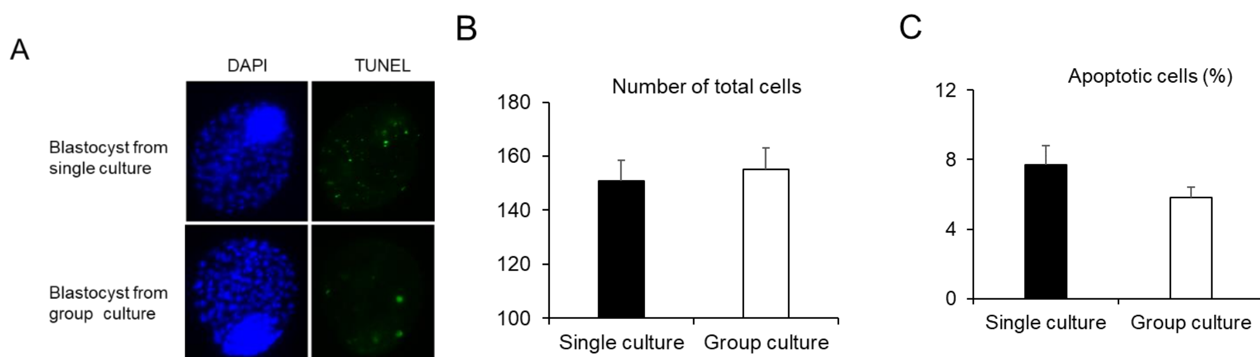

Figure 3: Effects of single vs. group culture methods during IVM on quality of blastocysts. Representative images of blastocysts at Day 8 (A) applied for TUNEL staining from single vs. group culture methods. TUNEL (green) and DAPI (blue) staining were used for apoptotic cells and DNA detection, respectively. Scale bar: 100 µm. The total cell number (B) and percentage of apoptotic cells (C) in each blastocyst, respectively obtained from single vs. group culture methods. Values are means ± S.E.M. from five independent experiments. DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Discussion

The present study examined the effect of culture methods single vs. group in oocyte maturation, fertilization and embryo development *in vitro*. First, the maturation rate of oocyte cultured in single vs. group IVM was investigated based on nuclear stage of the oocyte. The metaphase I (MI)-stage of oocytes evidenced as the alignment of paired homologous chromosomes along a single plane in the center of the cell; while the metaphase II (MII)-stage of oocytes marked the alignment of single chromosomes along a single plane in the center of the cell. The oocytes at the MII stage are *the standard stage of development that is required for* fertilization. Compared with the group method, the oocyte matured in the single IVM method showed many oocytes at the MI stage. The probable cause of this lower oocyte competence is the lake of paracrine system exists in single culture systems. In group culture systems, paracrine signaling influences the secretion of many metabolites from cumulus cells and oocytes, which is essential for oocytes' developmental acquisition. Therefore, oocytes undergoing the single culture method did not reach the MII stage of development, and the final maturation rate was decreased. More evidently, in this study, the lower maturation rate of oocyte derived single culture method is consistent with the lower rate of cumulus cell expansion in this group. These results were in accordance with those of previous studies reported by porcine (Gomez *et al*., 2012) and mice (Nishio *et al*., 2014). Previous studies also demonstrated the mechanisms of oocyte meiotic resumption with cumulus expansion and suggested that the expansion of cumulus cells is important for the cytoplasmic maturation of oocytes (Chen *et al*., 1993; Isobe & Terada, 2001; Larsen *et al*., 1996; Larsen *et al*., 1986). So, if the cumulus expansion was limited, oocyte maturation would be prevented, and the fertility and developmental competence of the oocyte would be decreased (Chen *et al*., 1993; Gomez *et al*., 2012).

In the present study, there was no significant difference in the rate of two-pronuclei formation between groups, as only matured oocytes were subjected to fertilization. Likewise, a study in mice also reported that despite, the total blastocysts rate significantly decreasing but number of pronuclei formation did not differ between single and group culture methods (Nishio *et al*., 2014). In contrast, the discrimination result was previously reported by (Choi *et al*., 2013), and the preantral follicles cultured in single drop dish culture methods decreased. In the present study, the developmental competence of the embryo at the two-cell stage was between the two culture methods (Table 2); however, the rate of total blastocyst formation in the single culture method was significantly reduced. This finding is consistent with previous *in vitro* studies in different species (Nishio *et al*., 2014; Sherbahn *et al*., 1996; van de Sandt *et al*., 1990; Vanhoutte *et al*., 2009). Together, these results indicated that there were some stimulus factors secreted from the surrounding oocytes during IVM or embryos during IVC in the group culture method, which is essential for embryos to reach the blastocyst stage. This study also demonstrated that the total cell number of blastocysts in the single culture method was not significantly different from that observed in the group culture method. Indeed, the total cell number of blastocysts increased during the period of blastocyst expansion and hatching (Sherbahn *et al*., 1996). It is generally accepted that the number of cells in blastocysts is an important indicator of embryo quality. The nobility of this study is the quality of the blastocysts derived from single vs. group methods are similar, even their developmental rate is varying between the groups.

Conclusion

Our results suggest that the maturation and developmental competence of oocytes were lower in the single-culture method than group-culture method. In contrast, the quality of blastocysts derived from the two methods of culture did not differ significantly. The final outcome of IVF success is associated with embryo transfer to the recipient and produced viable offspring. Thereby, the improvement of IVP-derived embryo quality has gained more attention from researchers. In addition, the single culture method is safer, more useful for infertility treatment and fertility preservation; and practiced in human IVM clinics. This finding represents a window of opportunity to reduce the constraints of group culture and produce quality embryo in bovine. However, further studies are needed to investigate the rate of post implantation development and fetal viability following transfer embryo derived by single culture method.

Acknowledgments

The authors acknowledge and thank their departments and universities.

Contribution by Authors

Conflict of Interests

There is no conflict of interest.

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