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Bone Marrow-Derived Mesenchymal Stem Cell-Conditioned Medium Supplementation Improves *In Vitro* Maturation and Subsequent Embryonic Development of Buffalo Oocytes

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JZ and YO designed the study. JZ did the experiments and drafted the manuscript. MO and YL edited the manuscript and analyzed the data. JS assisted in drafting the manuscript.

Key words
Buffalo, Bone marrow-derived mesenchymal stem cells, Conditioned medium, *In vitro* maturation, Oocytes

ABSTRACT

Previous studies have shown that conditioned medium of bone marrow-derived mesenchymal stem cells (BMSCs) improved in vitro maturation (IVM) of mouse oocytes. However, the effects of BMSC-conditioned medium (BMCM) on IVM and subsequent embryonic development of buffalo oocytes remain unclear. In this study, we examined the impacts of BMCM on maturation efficiency and embryonic development of buffalo oocytes after parthenogenetic activation. The results showed that BMCM that was collected on day 3 and added to IVM medium at a 10% addition concentration enhanced IVM and subsequent parthenogenetic development of buffalo oocytes. The present study serves a basis of obtaining high quality recipient oocytes for buffalo somatic cell nuclear transfer.

INTRODUCTION

Oocyte *in vitro* maturation (IVM) is one of the most important steps in somatic cell nuclear transfer for *in vitro* cloned embryo production. However, *in vitro* culture conditions, the isolation of oocytes from ovarian follicles can cause spontaneous resumption of meiosis. Cytoplasmic maturation commonly lags behind nuclear maturation in vitro, leading to poor IVM and developmental competence of oocytes (Jaroudi *et al*., 1999; Trounson, 2001; Mtango *et al*., 2003; Thomas *et al*., 2004; Kala *et al*., 2017; Xu *et al*., 2018). Success oocyte IVM depends on culture conditions and medium composition (Downs and Mastropolo, 1997; Nie *et al*., 2020). Therefore, optimization of IVM systems is warranted.

Bone marrow-derived stem cells (BMSCs) are a kind of adult stem cells with the potential to maintain self-renewal, proliferation, and multi-differentiation potential, and have become a focus of stem cell research in recent years (Bruder *et al*., 1998; Wang *et al*., 2015; Bornes *et al*., 2017; Gabrielyan *et al*., 2017; Liu *et al*., 2017; Zhang *et al*., 2017; He *et al*., 2018; Lin *et al*., 2019). In clinical research, BMSCs are used in studies of various disease treatment main through the secretion of cytokines to function as nutritional support, immune regulation and improvement the local microenvironment (Lee *et al*., 2010). As far as we know, there have few reports with respect to the application of BMSCs for oocyte IVM. Thus, the study focusing on the effects of BMSCs on IVM of oocytes is needed.

It is well known that BMSCs secret a variety of cytokines and growth factors, and some of these secreted bioactive factors could improve meiotic maturation in vitro and subsequent embryo development capacity (Akbari *et al*., 2017; Bezerra *et al*., 2019). Previous studies have shown that BMSC-conditioned medium (BMCM) promoted preantral follicle growth, oocyte maturation, and sequential embryonic development in mice (Ling *et al*., 2008). Also, many reports have demonstrated that supplementation with BMCM during mouse oocyte IVM enhanced their maturation and fertilization rates, and subsequent preimplantation embryo development (Kalehoei *et al*., 2022). In addition, co-culture with BMSCs improved the survival rates, increased the growth velocity, and enhanced the viability of human preantral follicles in a dose-dependent manner (Xia *et al*., 2015). Based on the above investigations, further systematic studies with respect to the effects of BMSCs on oocyte IVM need to

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undergo in the future.

As one of the leading domestic animals in southern China, buffalo (*Bubalus bubalis*) is widely used for plough, meat, and milk (Lu *et al*., 2018; Luo *et al*., 2020; Chen *et al*., 2022; Tong *et al*., 2022; Yuan *et al*., 2022). To our knowledge, there is little information available in the literature with respect to the effects of BMCM on IVM and developmental competence of buffalo oocytes. In the present study, we creatively investigated whether the addition of BMCM could improve IVM and subsequent embryonic development of buffalo oocytes. We first examined the changes in the first polar body extrusion (PB1) and then assessed subsequent development potential, including oocyte cleavage and blastocyst formation of buffalo oocytes after parthenogenetic activation (PA). Our study provides a new insight into how BMSCs affecting IVM of buffalo oocytes, and it also offers a novel approach for the procurement of high-quality recipient oocytes for buffalo somatic cell nuclear transfer.

MATERIALS AND METHODS

Reagents and culture medium

All of the chemical reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), except for tissue culture medium 199 (TCM199) and fetal bovine serum (FBS), which were purchased from Gibco (Carlsbad, CA). The IVM medium was TCM199 supplemented with 26.2 mM $NaHCO_3$, 5 mM HEPES, 0.1 μg/mL FSH, 5% FBS, 60 mg/L penicillin, and 100 mg/L streptomycin sulphate. The embryo culture medium (CM) was TCM199 supplemented with 3% FBS. The complete cell medium for buffalo BMSCs contained low-glucose Dulbecco's modified Eagle's medium (DMEM) and was supplemented with 10% FBS, 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin.

Isolation and culture of BMSCs

Buffalo (*Bubalus bubalis*) fetuses were collected from a local commercial slaughterhouse and transported to the laboratory at 37°C in physiological saline (0.9% NaCl) within 4 h, then were disinfected with 75% ethanol and cleaned with physiological saline for 2-3 times. Buffalo BMSCs were isolated from limbs' long bone marrow cavity of buffalo fetuses, whose body length ranging from 10 cm to 18 cm, with the total bone marrow adherent method and cultured in the complete cell medium. When buffalo BMSCs were cultured for 24 h, the cell medium was replaced with fresh cell medium. When buffalo BMSCs reached 80% to 90% confluency, they were passaged or frozen for later experiments. Buffalo BMSCs were cultured in normoxic conditions under a humidified atmosphere of 5% CO_2 in air at 38.5°C. In order to maintain the consistency of the experimental results, except for individual experiments, buffalo BMSCs at passage 3 were used as the cell resources for all experiments in this study.

Collection of BMCM

Buffalo BMSCs at passage 3 were cultured up to 80% confluency in 12-well plates, washed three times with PBS, and incubated with culture medium (low-glucose DMEM + 10% FBS, with 1 mL medium added per well) at 37°C with 100% humidity and 5% CO_2. BMCM was collected at day 0, 1, 2, 3, and 4 of culture (1 mL per well) and centrifuged at 300 × g for 10 min. The culture medium was not replaced during the process of collecting BMCM. The conditioned medium was then filtered through a 0.2-μm membrane and stored for less than 2 weeks at 4°C until further use. During the process of IVM, buffalo BMCM was added to the IVM medium.

Collection and IVM of oocytes

Buffalo ovaries were collected and cleaned as the above procedure in the isolation and culture of GCs. Buffalo cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles (2-6 mm in diameter) with an 18-gauge needle attached to a disposable 10-mL syringe, and then washed twice with cell-cleaning medium. COCs with at least 2 intact cumulus cell (CC) layers were selected and placed in maturation medium at 38.5°C in a 100% humidified atmosphere of 5% CO_2 for 24 h.

PA of oocytes

After IVM, the denuded oocytes were treated with 5 μM ionomycin in CM for 5 min, followed by 2 mM 6-dimethylaminopurine for 4 h to activate oocytes parthenogenetically. After PA, approximately 15 oocytes were co-cultured with CC monolayers in 25-μL CM droplets overlaid with mineral oil at 38.5°C in 100% humidity and 5% CO_2. The CM medium was replaced every 48 h by using the method of medium replacement with a 50% aliquot of fresh medium. Cleavage of oocytes was verified after 24 h, and blastocyst formation rate was evaluated within 8 days.

In vitro differentiation and staining identification

When buffalo BMSCs reached 80% to 90% confluency, the osteoblast induction medium was added to perform osteoblast induction according to the manufacturer's instructions (Gibco, Carlsbad, USA). The induction medium was replaced every three days, and the staining identification was performed after 21 days of continuous induction. The staining identification of osteoblast induction was detected using Alizarin Red Detection Kit, and the staining identification of adipogenic induction was identified using Oil Red Detection Kit

according to the manufacturer's instructions. The chondrocytes induction was also referred to as osteoblast and adipocytes induction's procedure, and the staining identification was detected using Alcian Blue Kit according to the Kit's instruction. Adipogenic induction procedure was referred to osteoblast induction's method, and the staining identification was performed after 14 days of continuous induction.

Experimental design

Two experiments were designed. In the first experiment the effects of BMCM collection time was determined on IVM and subsequent parthenogenetic development of buffalo oocytes. The aim of this experiment was to explore the optimal collection time of BMCM. Buffalo COCs with at least 2 intact CC layers were placed into maturation medium supplementation with BMCM recovered at different collection times (1 day, 2 days, 3 days, and 4 days) for 24 h, and we assessed PB1 extrusion rate, cleavage rate and blastocyst formation rate to select the optimal BMCM collection time. Each treated group was supplemented with 10% BMCM to maintain consistency across experimental results. This experiment was repeated at least 5 times.

In the second experiment the effects of BMCM addition concentration was determined on IVM and subsequent parthenogenetic development of buffalo oocytes. The objective of this experiment was to investigate the optimal addition concentration of BMCM. Buffalo COCs with at least 2 intact CCs were placed into maturation medium supplementation with BMCM in different concentrations (5%, 10%, 20%, and 40%) for 24 h, and then, we evaluated PB1 extrusion rate, cleavage rate and blastocyst formation rate to select the optimal addition concentration of BMCM. Each treated group was supplemented with BMCM recovered at 3 days to maintain the consistency over experimental results. This experiment was repeated at least 5 times.

Statistical analysis

All data were presented as the mean ± standard error (SE). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Student's *t* test. All statistical analyses were performed by using SPSS 22.0 software. p values < 0.05 were deemed to be significant.

RESULTS

Isolation, culture and identification of buffalo BMSCs

Buffalo BMSCs were isolated from bone marrow cavity of buffalo fetuses ranging from 10 cm to 18 cm in body length (Fig. 1A). Primary buffalo BMSCs aggregated into helical colonies at bottom of plates and exhibited typical fibroblast-like morphology, indicating their BMSC origin (Fig. 1B). Buffalo BMSCs still showed the original morphological features of BMSCs when passaged three times (Fig. 1C). Upon identification, buffalo BMSCs exerted high alkaline phosphatase activity and could be induced into osteoblasts, chondrocytes, and adipocytes (Fig. 1D). These results demonstrated that BMSCs isolated from buffalo fetuses in the present study were purified BMSCs, which were then used for subsequent experiments.

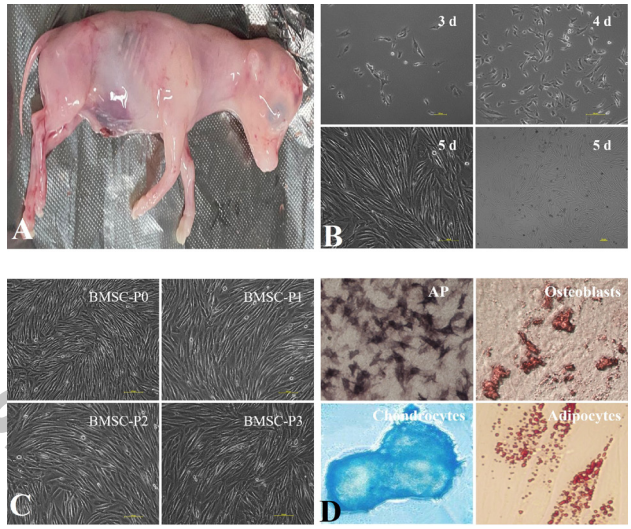

Fig. 1. Isolation, culture and identification of buffalo BMSCs: (A) Buffalo fetuses ranging from 10 cm to 18 mm in body length used to isolate buffalo BMSCs. (B) The morphology of primary buffalo BMSCs on day 3, 4 and 5. (C) The morphology of buffalo BMSCs from passage 1 to passage 3. (D) Alkaline phosphatase live staining of buffalo BMSCs; Osteogenic, chondrogenic, and adipogenic induction differentiation of buffalo BMSCs. Scale bars = 100 μm.

Effect of BMCM collection time on IVM and subsequent parthenogenetic development of buffalo oocytes

The effects of BMCM collection time on the maturation rate of buffalo oocytes were evaluated by the rate of PB1 extrusion, and BMCM effects on buffalo oocyte developmental competence were analysed by assessing the cleavage rate and blastocyst formation rate of parthenogenetically activated embryos. The results showed that supplementation with BMCM collected at 2 days or 3 days significantly improved the maturation rate of buffalo oocytes versus control (47.2±0.52%, 49.3±0.46% vs. 44.3±0.41%, respectively ($p < 0.05$). In addition, the 3-day group exerted significant higher maturation rate of buffalo oocytes than the 2-day group (49.3%±0.46% vs. 47.2%±0.52%; $p < 0.05$) (Table I).

Table I. Effect of BMCM collection time on IVM of buffalo oocytes.

Collection times	Replicates	Number of oocytes	Number of matured oocytes	Rates of maturation (mean ± SE)
Control	6	273	121	44.3 ± 0.41%^{c}
1 day BMCM	6	271	122	45.1 ± 0.55%^{c}
2 days BMCM	6	259	122	47.2 ± 0.52%^{b}
3 days BMCM	6	276	136	49.3 ± 0.46%^{a}
4 days BMCM	6	279	125	44.9 ± 0.62%^{c}

Note: Values in the same column with different superscript letters differ significantly by ANOVA ($p < 0.05$).

The addition of BMCM on days 2 or 3 significantly promoted the cleavage rate (75.4 ± 0.38%, 80.5 ± 0.71% vs. 70.5 ± 0.79%, respectively; $p < 0.05$) and blastocyst formation rate (35.2 ± 1.20%, 40.1% ± 0.97 vs. 30.2 ± 0.77%, respectively; $p < 0.05$) of PA embryo when compared with the control group. Besides, the group of BMCM collected at day 3 had significant higher cleavage rate (80.5 ± 0.71% vs. 75.4 ± 0.38%; $p < 0.05$) and blastocyst formation rate (40.1 ± 0.97% vs. 35.2 ± 1.20%; $p < 0.05$) of PA embryo than the group of BMCM collected on day 2 (Table II). These results suggested that IVM supplementation with BMCM on day 2 or 3 enhanced IVM rate and embryo developmental competence of buffalo oocytes after PA, and BMCM collection time of day 3 was the optimum.

Effect of BMCM addition concentration on IVM and subsequent parthenogenetic development of buffalo oocytes

We assessed the effects of BMCM addition concentration on IVM and developmental potential of buffalo oocytes. Our results showed that the addition of 5%, 10% or 20% BMCM significantly promoted the maturation rate of buffalo oocytes versus control (46.5±0.55%, 50.2±0.59%, 47.2±0.88% vs. 44.1±0.29%, respectively; $p < 0.05$). Additionally, the 10%-addition group exhibited significant higher maturation rate of buffalo oocytes than other treatment groups (50.2±0.59% vs. 46.5±0.55%, 47.2 ± 0.88%, respectively; $p < 0.05$) (Table III).

Supplementation with 5%, 10% or 20% BMCM significantly enhanced the cleavage rate (74.2 ± 0.71%, 79.0 ± 0.74%, 74.9 ± 0.26% vs. 69.4 ± 0.33%, respectively; $p < 0.05$) and blastocyst formation rate increased (35.0 ± 0.47%, 38.8 ± 0.42%, 35.2 ± 0.21% vs. 29.2% ± 0.56%, respectively ($p < 0.05$) of PA embryos when compared with the control group. Furthermore, IVM with addition concentration of 10% had significantly higher cleavage rate (79.0 ± 0.74% vs. 74.2 ± 0.71%, 74.9 ± 0.26%, respectively; $p < 0.05$) and blastocyst formation rate (38.8 ± 0.42 vs. 35.0 ± 0.47%, 35.2 ± 0.21%, respectively; $p < 0.05$) than other experimental groups (Table IV). These results demonstrated that the addition of 5%, 10% or 20% BMCM improved IVM rate and embryo developmental competence of buffalo oocytes after PA, and BMCM addition concentration of 10% was the best.

Table II. Effect of BMCM collection time on IVM subsequent parthenogenetic development of buffalo oocytes.

Collection times	Replicates	Number of oocytes	Number of cleavage (mean ± SE)	Number of blastocyst (mean ± SE)
Control	6	203	143(70.5±0.79%)^{c}	61(30.2±0.77%)^{c}
1 day BMCM	6	197	143(72.3±1.23%)^{c}	65(32.9±0.50%)^{bc}
2 days BMCM	6	191	144(75.4±0.38%)^{b}	67(35.2±1.20%)^{b}
3 days BMCM	6	200	161(80.5±0.71%)^{a}	80(40.1±0.97%)^{a}
4 days BMCM	6	199	148(74.5±1.54%)^{b}	66(33.3±0.74%)^{bc}

Note: Values in the same column with different superscript letters differ significantly by ANOVA ($p < 0.05$).

Table III. Effects of BMCM addition concentration on IVM of buffalo oocytes.

Addition concentrations	Replicates	Number of oocytes	Number of matured oocytes	Rates of maturation (mean ± SE)
Control	5	257	113	44.1 ± 0.29%^{c}
5% BMCM	5	227	106	46.5 ± 0.55%^{b}
10% BMCM	5	228	114	50.2 ± 0.59%^{a}
20% BMCM	5	228	107	47.2 ± 0.88%^{b}
40% BMCM	5	237	107	45.1 ± 0.24%^{bc}

Note: Values in the same column with different superscript letters differ significantly by ANOVA ($p < 0.05$).

Table IV. Effects of BMCM addition concentration on IVM subsequent parthenogenetic development of buffalo oocytes.

Addition concentrations	Replicates	Number of oocytes	Number of cleavage (mean ± SE)	Number of blastocyst (mean ± SE)
Control	5	251	174 (69.4 ± 0.33%) [c]	73 (29.2 ± 0.56%) [c]
5% BMCM	5	221	164 (74.2 ± 0.71%) [b]	77 (35.0 ± 0.47%) [b]
10% BMCM	5	222	176 (79.0 ± 0.74%) [a]	86 (38.8 ± 0.42%) [a]
20% BMCM	5	216	162 (74.9 ± 0.26%) [b]	76 (35.2 ± 0.21%) [b]
40% BMCM	5	229	163 (71.3 ± 0.55%) [c]	71 (31.9 ± 0.73%) [c]

Note: Values in the same column with different superscript letters differ significantly by ANOVA ($p < 0.05$).

DISCUSSION

BMSCs are adult stem cells usually obtained from bone marrow cavity, which are attractive candidates for cell-based therapeutic strategies, primarily because of their intrinsic ability to re-renew and undergo multipotential differentiation as well as being amenable to genetic manipulation (Deans and Moseley, 2000; Kassem *et al*., 2004; Caplan and Dennis, 2006). It is generally accepted that BMSCs secrete a variety of cytokines and growth factors, such as MCP-1, VEGF-A, EGF, FGF-2, IL-6, LIF, and TGF-β (Heil *et al*., 2004; Caplan and Dennis, 2006). Since it is well known that cytokines and growth factors stimulate meiotic progress and the processes associated with IVM, the effects of BMSCs in the form of conditioned medium on IVM and subsequent embryonic development of oocytes are explored in this study.

In the present study, we investigated for the first time the influences of BMCM added to the maturation medium on IVM and subsequent parthenogenetic development of buffalo oocytes. The results showed that BMCM (3 days and 10%) not only improved the oocyte maturation rate, but also promoted the subsequent embryonic development of buffalo oocytes after PA. As too long collection time of BMCM may lead to poor cellular status of BMSCs, while too high additional concentration of BMCM may cause excessive effects on IVM, appropriate collection time and addition concentration of BMCM are crucial to IVM of buffalo oocytes. These findings were not exactly consistent with results of recent reports that granulosa cell-conditioned medium and theca cell-conditioned medium added to maturation medium affected IVM and development competence of buffalo oocytes after PA (Zhang *et al*., 2020, 2022). This difference in findings between BMSCs and granulosa cells or theca cells may be a reflection of the differences in cell types and secretory characteristics.

Oocyte maturation includes both nuclear and cytoplasmic maturation, and poor developmental potential of IVM oocytes is caused by incorrect cytoplasmic maturation despite completion of nuclear maturation (Cha and Chian, 1998; Marchal *et al*., 2001; Salamone *et al*., 2001; Ruddock *et al*., 2004; Tao *et al*., 2008; Reichman *et al*., 2010). Our study demonstrated that BMCM enhanced both IVM and subsequent parthenogenetic development of buffalo oocytes. These results indicated that BMCM improved IVM and subsequent development competence principally by stimulating nuclear maturation and strengthening cytoplasmic maturation, thereby promoting synchrony between nuclear and cytoplasmic maturation in buffalo. Our results were in accordance with the findings of the previous report that the effects of BMCM on IVM and development potential of mouse oocytes (Ling *et al*., 2008; Kalehoei *et al*., 2022).

Although BMCM exerts positive effects on IVM and subsequent embryonic development of buffalo oocytes, the detailed mechanism of BMSM affecting IVM of buffalo oocytes remains unclear. Further research is still needed to fully evaluate the action of BMCM on oocyte IVM in buffalo, especially focusing on the essential cytokines and growth factors which are not only specific secretion of BMSCs, but also necessary for oocyte maturation *in vitro*.

CONCLUSIONS

In conclusion, BMCM (3 days and 10%) not only improved IVM efficiency, but also promoted subsequent embryo development of buffalo oocytes after PA. This study serves as a foundation to acquire high quality recipient oocytes for buffalo somatic cell nuclear transfer.

DECLARATIONS

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IRB approval

This study was approved and monitored by the Animal Experiment Institutional Review Board (IRB) of Guangxi Medical University, Nanning, China.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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