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Original Article

Effect of supplement of SCM in culture medium for *in vitro* development of bovine *in vitro* fertilized oocytes

Sang Jun Uhm*

Department of Animal Science, Sangji University, Wonju 26339, Korea

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***Correspondence**
Sang Jun Uhm
E-mail: sjuhm@sangji.ac.kr

Author's Position and Orcid no.
Uhm SJ, Professor,
https://orcid.org/0000-0002-9573-2831

ABSTRACT

Background: The successful production of superior or transgenic offspring from *in vitro* produced embryos in cattle relies heavily on the quality of blastocyst stage embryos. In order to enhance the developmental competency of these embryos, a novel culture method was devised.

Methods: This study utilized stem cell culture medium (SCM) from hESCs as a supplement within the culture medium for bovine *in vitro* produced embryos. To gauge the efficacy of this approach, *in vitro* fertilized embryos were subjected to culture in CR1aa medium enriched with one of three supplements: 0.3% BSA, 10% FBS, or 10% SCM.

Results: The blastocyst development and hatching rates of one-cell zygotes cultured in CR1aa medium supplemented with SCM (23.9% and 10.2%) surpassed those cultured in CR1aa medium supplemented with BSA (9.3% and 0.0%) or FBS (3.1% and 0.0%) ($p < 0.05$). Furthermore, post-zygotic gene activation, cleaved embryos cultured in CR1aa medium supplemented with SCM (57.8% and 34.5%) exhibited notably higher rates ($p < 0.05$) compared to those cultured with BSA (12.9% and 0.0%) or FBS (45.7% and 22.5%) supplementation. Furthermore, the microinjection of SCM into the cytoplasm or pronucleus of fertilized zygotes resulted in elevated blastocyst development and hatching rates, particularly when the microinjected embryos were subsequently cultured in CR1aa medium supplemented with SCM from the 8-cell embryo stage onwards ($p < 0.05$), in contrast to those cultured with FBS supplementation.

Conclusions: In conclusion, this study conclusively demonstrated that the incorporation of SCM into the culture medium significantly enhances the developmental progress of preimplantation embryos.

Keywords: bovine, bovine serum albumin, fetal bovine serum, *in vitro* fertilized zygote, stem cell culture medium

INTRODUCTION

The first transgenic mice in 1982 by microinjection into the pronucleus of fertilized mouse oocytes (Palmiter et al., 1982) produced in biotechnology-based research. Since, somatic cell nuclear transfer (Schnieke et al., 1997), viral vector system (Chan et al., 1998) and sperm vector system (Lavitrano et al., 1989) have been developed to

introduction of foreign genes into eggs for production of transgenic animals. Especially, research on producing transgenic dairy cows that secrete large amounts of milk for the production of new biopharmaceutical proteins has been attempted by many researchers for a long time (Bowen et al., 1994; Han et al., 1996). To produce these transgenic cows, embryos at the blastocyst stage are transferred to surrogate mothers (Han et al., 1996; Richt et al., 2007; Yang et al., 2011; Xu et al., 2013; Wu et al., 2015; Su et al., 2016). The successful offspring production by fertilized embryo transfer depends on several factors on the conception rate. Choe et al. (2010) pointed out three main causes, namely the quality of fertilized eggs, the skill of embryo transferers, and the condition of fertilized cattle, as the effect on the conception rate. Therefore, the quality of embryos of blastocyst stage is important for the successful production of offspring. To this end, various additives such as fetal bovine serum (FBS), antioxidants, and growth factors were added to the *in vitro* culture medium for embryonic development (Larson et al., 1992; Lima et al., 2006; Lee et al., 2011; Arat et al., 2016; Ramos-Deus et al., 2020).

Recently, embryonic stem cell (ESC) conditioned medium has been used for embryo development. Addition of mouse ESC conditioned medium improved *in vitro* maturation of porcine oocytes and affected embryonic development ability (Kim and Park, 2019). Also, Miraki et al. (2017) reported that ESC conditioned medium supported the *in vitro* maturation of mouse oocytes, and the blastocysts produced after fertilization were transplanted into surrogate mothers and gave birth to normal healthy offspring. ESC is the pluripotent stem cell derived from the inner cell mass of the blastocyst. An advanced mass spectrometry approach has revealed the presence of many growth factors such as EGF, IGF-1, IGF-2 and SCF in the culture of human embryonic stem cells (Bendall et al., 2009). ESCs are expected to be a better supplement source in conditioned media for *in vitro* maturation of oocytes and *in vitro* culture of embryos. Therefore, this study hypothesized that ESC conditioned medium could be a good source of various soluble factors for *in vitro* development of bovine embryos of *in vitro* matured and fertilized oocytes. However, reports on the possible usefulness of ESC conditioned medium for bovine embryos are currently lacking.

Thus, it was conducted to develop an optimal serum-free culture method for *in vitro* culture of bovine transgenic embryos as well as *in vitro* fertilized zygotes in order to increase the conception rate after transplantation of embryos and the successfully production of calves. For this, the present study was designed to investigate the suitability of ESC conditioned medium as a source of complex soluble factors or as a replacement of FBS in the bovine embryo culture medium. Furthermore, this study was conducted to solve the problems of low pregnancy rate and offspring production in the production of transgenic animals by improving the quality of blastocysts in cattle.

MATERIALS AND METHODS

Oocyte retrieval and *in vitro* maturation (IVM)

Bovine ovaries were collected from a local abattoir and transported to the laboratory in saline maintained at 30-37℃. Cumulus-oophorus-complexes (COCs) were aspirated from medium-sized follicles (2-5 mm diameter) using 10 mL syringe fitted with an 18 G needle. The COCs were matured in groups of 50 in 500 μL of Tissue Culture Medium 199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY) supplemented with 25 mM $NaHCO_3$, 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), 5 μg/mL Folltropin V (Vetrepharm, Canada), and 1 μg/mL estradiol 17-β under mineral oil at 39℃ in a humidified atmosphere of 5% CO_2 in air for 24 h.

In vitro fertilization and development

In vitro fertilization of matured oocytes were washed three times with fertilization medium (Fert-TALP medium containing 1 mM caffeine sodium benzoate and 0.3% [v/v] BSA), and were placed in groups of 10 to 15 oocytes per 50 μL droplets of fertilization medium under mineral oil. For bovine sperm, KPN frozen semen was used, and the thawed semen was mixed with BO medium containing 1 mM caffeine sodium benzoate and 0.1% (v/v) BSA, centrifuged, and washed by collecting concentrated sperm located in the lower layer. *In vitro* matured oocytes were *in vitro* fertilized with frozen-thawed semen (1×10^6/mL) in fertilization medium medium for 18 h. *In vitro* fertilized zygotes were cultured in CR1aa medium supplemented with 0.3% fatty acid free BSA during day 2 then only cleaved embryos were transferred to CR1aa medium supplemented with each concentrations of 10% FBS or SCM (stem cell culture medium) then cultures until blastocyst and hatched blastocyst stages. All procedure was conducted at 39℃ in a

humidified atmosphere of 5% CO_2 in air.

Preparation of SCM

SCM for culture of hESCs were provided by professor Hyung-Min Chung in Konkuk University of Republic of Korea. The SCM was prepared from hESCs as per a previously described protocol (Yang et al., 2019), with modifications. Briefly, hESCs (WA09, WiCell) were cultured in Stem MACS™ iPS-Brew XF (MACS; Miltenyi Biotec; Cat# 130-104-368) on culture plates coated with hESC-qualified Matrigel™ (Corning) at 37℃ in a humidified atmosphere of 5% CO_2 in the air. SCM was filtered and dispensed with a 0.22 μm millipore filter, and stored frozen at -80℃ in small amounts before use.

Experimental designs

Experiment 1 was studied that the fertilized zygotes were examined the developmental ability in each CR1aa medium supplemented with 0.3% BSA, 10% FBS or 10% SCM at 18 h after *in vitro* fertilization of matured oocytes.

Experiment 2 was studied that the cleaved embryos were examined the developmental ability in each CR1aa medium supplemented with 0.3% BSA, 10% FBS or 10% SCM on day 3 after *in vitro* fertilization of matured oocytes.

Experiment 3 was studied that the microinjected zygotes of medium into cytoplasm of fertilized zygotes at 18 h after *in vitro* fertilization of matured oocytes were examined the developmental ability in each CR1aa medium supplemented with 10% FBS or SCM.

Experiment 4 was studied that the microinjected zygotes of medium into pronucleus of fertilized zygotes at 18 h after *in vitro* fertilization of matured oocytes were examined the developmental ability in each CR1aa medium supplemented with 10% FBS or SCM.

Statistical analyses

Chi-square of the SAS program was performed for statistical processing to test the significance of the experimental results between treatment groups, and only $p < 0.05$ or less were recognized as significant.

RESULTS

Experiment 1. *In vitro* development in each CR1aa medium supplemented with BSA, FBS or SCM of *in vitro* maturation and fertilization oocytes

The fertilized zygotes were cultured in each CR1aa medium supplemented with 0.3% BSA, 10% FBS or 10% SCM at 18 h after *in vitro* fertilization. As a result, as shown in Table 1, the cleavage rates as these fertilized zygotes were not significantly difference in each CR1aa medium supplemented with BSA (85.4 ± 2.4%), FBS (83.2 ± 1.8%) or SCM (84.1 ± 2.1%). The blastocyst and hatching rates from these cleaved embryos in CR1aa medium supplemented with SCM (23.9 ± 2.1% and 10.2 ± 2.4%) were significantly higher ($p < 0.05$) than those of cleaved embryos in each CR1aa medium supplemented with BSA (9.3 ± 1.7% and 0.0 ± 0.0%) or FBS (3.1 ± 1.8% and 0.0 ± 0.0%). Fig. 1 shows

Table 1. Developmental ability by supplement of BSA, FBS or SCM in CR1aa medium of fertilized zygotes at 18 h after *in vitro* fertilization

Treatment*	No. of zygotes fertilized	No. (%) of embryos cleaved	No. (%) of blastocysts/ cleaved embryos	No. (%) of hatching blastocysts/ cleaved embryos
BSA	254	217 (85.4 ± 2.4)[a]	20 (9.3 ± 1.7)[b]	0 (0.0 ± 0.0)[a]
FBS	254	211 (83.2 ± 1.8)[a]	7 (3.1 ± 1.8)[a]	0 (0.0 ± 0.0)[a]
SCM	254	214 (84.1 ± 2.1)[a]	51 (23.9 ± 2.1)[c]	22 (10.2 ± 2.4)[b]

*Supplement of 0.3% fatty acid free BSA, 10% FBS or 10% SCM in CR1aa medium.
Values with different superscripts ([a, b, c]) within column differ significantly ($p < 0.05$).

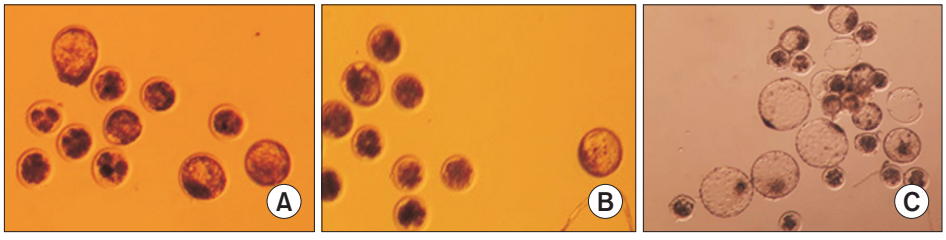

Fig. 1. Blastocyst formation at day 8 after *in vitro* culture of *in vitro* fertilized zygotes. The fertilized zygotes at 18 h after *in vitro* fertilization were cultured in each CR1aa medium supplemented with BSA, FBS or SCM (A: 0.3% fatty acid BSA, B: 10% FBS, C: 10% SCM).

the development of others blastocysts from these fertilized zygotes cultured in each CR1aa medium supplemented with BSA, FBS or SCM at day 8 after *in vitro* fertilization.

Experiment 2. *In vitro* development in each CR1aa medium supplemented with BSA, FBS or SCM of cleaved embryos on day 3 after *in vitro* fertilization of matured oocytes

The fertilized embryos were cultured in CR1aa medium supplemented with 0.3% BSA during day 3 after *in vitro* fertilization. The cleaved embryos on day 3 after *in vitro* fertilization were cultured in each CR1aa medium supplemented with 0.3% BSA, 10% FBS or 10% SCM. As a result, as shown in Table 2, the developmental rates of blastocysts from cleaved embryos were 12.9 ± 1.4%, 45.7 ± 2.6% or 57.8 ± 4.2% in each CR1aa medium supplemented with BSA, FBS or SCM. Also, the developmental rates of hatching blastocysts from cleaved embryos were 0.0 ± 0.0%, 22.5 ± 2.4% or 34.5 ± 3.4% in each CR1aa medium supplemented with BSA, FBS or SCM. However, the blastocyst and hatching rates of cleaved embryos cultured in CR1aa medium supplemented with FBS was significantly higher ($p < 0.05$) than those in CR1aa medium supplemented with BSA. Furthermore, the blastocyst and hatching rates of cleaved embryos cultured in CR1aa medium supplemented with SCM ware significantly higher ($p < 0.05$) than those in CR1aa medium supplemented with FBS.

Table 2. Developmental ability by supplement of BSA, FBS or SCM in CR1aa medium of cleaved embryos on day 3 after *in vitro* fertilization

Treatment*	No. of embryos	No. (%) of blastocysts	No. (%) of hatching blastocysts
BSA	194	25 (12.9 ± 1.4)[a]	0 (0.0 ± 0.0)[a]
FBS	194	89 (45.7 ± 2.6)[b]	44 (22.5 ± 2.4)[b]
SCM	194	112 (57.8 ± 4.2)[c]	67 (34.5 ± 3.4)[c]

*Cleaved embryos cultured in CR1aa medium supplemented with 0.3% fatty acid BSA during day 3 after *in vitro* fertilization were cultured in each CR1aa medium supplemented with 0.3% fatty acid BSA, 10% FBS or 10% SCM.

Values with different superscripts ([a, b, c]) within column differ significantly ($p < 0.05$).

Experiment 3. *In vitro* development in each CR1aa medium supplemented with FBS or SCM after microinjection of medium into cytoplasm of *in vitro* fertilized zygotes

In vitro fertilized zygotes were microinjected of medium into cytoplasm at 18 h after *in vitro* fertilization. Theses cytoplasmic microinjected zygotes of medium were cultured during day 2 in CR1aa medium supplemented with 0.3% BSA. The cleaved embryos on day 3 after *in vitro* fertilization were cultured in each CR1aa medium supplemented with 10% FBS or SCM. As a result, as shown in Table 3, the cytoplasmic microinjected zygotes (76.4 ± 2.1%) of medium were significantly lower ($p < 0.05$) cleavage rate than unmicroinjected zygotes (control; 85.9 ± 2.0%). In the blastocyst and hatching rates from cleaved embryos, those of cytoplasmic microinjected cleaved embryos (27.6 ± 2.1% and 13.3 ± 2.3%) cultured in CR1aa medium supplemented with FBS were significantly lower ($p < 0.05$) than those of control cleaved embryos (36.3 ± 2.1% and 20.3 ± 2.0%) cultured in CR1aa medium supplemented with FBS, and those of cleaved embryos (34.2 ± 2.9% and 18.9 ± 2.7%) cultured in CR1aa medium supplemented with SCM were significantly higher ($p < 0.05$) than those of cleaved embryos (27.6 ± 2.1% and 13.3 ± 2.3%) cultured in CR1aa medium supplemented with FBS. However, the blastocyst and hatching rates from cleaved embryos were not significantly difference between cytoplasmic microinjected cleaved embryos (34.2 ± 2.9% and

Table 3. Developmental ability by supplement of FBS or SCM in CR1aa medium after microinjection of medium into cytoplasm of *in vitro* fertilized zygotes

Treatment*		No. of zygotes fertilized	No. (%) of embryos cleaved**	No. (%) of blastocysts/ cleaved embryos	No. (%) of hatching blasto-cysts/cleaved embryos
Control	BSA-FBS	256	220 (85.9 ± 2.0)[a]	80 (36.3 ± 2.1)[a]	45 (20.3 ± 2.0)[a]
Cytoplasm injection	BSA-FBS	512	392 (76.4 ± 2.1)[b]	54/196 (27.6 ± 2.1)[b]	26 (13.3 ± 2.3)[b]
	BSA-SCM			67/196 (34.2 ± 2.9)[a]	37 (18.9 ± 2.7)[a]

*Cleaved embryos cultured in CR1aa medium supplemented with 0.3% fatty acid BSA during day 3 after *in vitro* fertilization were cultured in each CR1aa medium supplemented with 10% FBS or SCM.

**Each half of cytoplasmic microinjected cleaved embryos of medium were cultured in each CR1aa medium supplemented with FBS or SCM.

Values with different superscripts ([a, b]) within column differ significantly ($p < 0.05$).

Table 4. Developmental ability by supplement of FBS or SCM in CR1aa medium after microinjection of medium into pronucleus of *in vitro* fertilized zygotes

Treatment*		No. of zygotes fertilized	No. (%) of embryos cleaved**	No. (%) of blastocysts/ cleaved embryos	No. (%) of hatching blastocysts/cleaved embryos
Control	BSA-FBS	198	169 (84.8 ± 2.4)[a]	60 (35.5 ± 3.7)[a]	34 (19.9 ± 3.3)[a]
Pronucleus injection	BSA-FBS	398	282 (70.8 ± 2.4)[b]	38/141 (26.9 ± 2.5)[b]	16 (11.4 ± 2.0)[b]
	BSA-SCM			47/141 (33.4 ± 2.7)[a]	25 (17.8 ± 3.3)[a]

*Cleaved embryos cultured in CR1aa medium supplemented with 0.3% fatty acid BSA during day 3 after *in vitro* fertilization were cultured in each CR1aa medium supplemented with 10% FBS or SCM.

**Each half of pronucleus microinjected cleaved embryos of medium were cultured in each CR1aa medium supplemented with FBS or SCM.

Values with different superscripts ([a, b]) within column differ significantly ($p < 0.05$).

18.9 ± 2.7%) cultured in CR1aa medium supplemented with SCM and control cleaved embryos (36.3 ± 2.1% and 20.3 ± 2.0%) cultured in CR1aa medium supplemented with FBS.

Experiment 4. *In vitro* development in each CR1aa medium supplemented with FBS or SCM after microinjection of medium into pronucleus of *in vitro* fertilized zygotes

In vitro fertilized zygotes were microinjected of medium into pronucleus at 18 h after *in vitro* fertilization. Theses pronucleus microinjected zygotes of medium were cultured during day 2 in CR1aa medium supplemented with 0.3% BSA. The cleaved embryos on day 3 after *in vitro* fertilization were cultured in each CR1aa medium supplemented with 10% FBS or SCM. As a result, as shown in Table 4, the pronucleus microinjected zygotes (70.8 ± 2.4%) of medium were significantly lower ($p < 0.05$) cleavage rate than unmicroinjected zygotes (control; 84.8 ± 2.4%). In the blastocyst and hatching rates from cleaved embryos, those of pronucleus microinjected cleaved embryos (26.9 ± 2.5% and 11.4 ± 2.0%) of medium cultured in CR1aa medium supplemented with FBS were significantly lower ($p < 0.05$) than those of control cleaved embryos (35.5 ± 3.7% and 19.9 ± 3.3%) cultured in CR1aa medium supplemented with FBS, and those of cleaved embryos (33.4 ± 2.7% and 17.8 ± 3.3%) cultured in CR1aa medium supplemented with SCM were significantly higher ($p < 0.05$) than those of cleaved embryos (26.9 ± 2.5% and 11.4 ± 2.0%) cultured in CR1aa medium supplemented with FBS. However, the blastocyst and hatching rates from cleaved embryos were not significantly difference between pronucleus microinjected cleaved embryos (33.4 ± 2.7% and 17.8 ± 3.3%) cultured in CR1aa medium supplemented with SCM and control cleaved embryos (35.5 ± 3.7% and 19.9 ± 3.3%) cultured in CR1aa medium supplemented with FBS.

DISCUSSION

In biotechnology research, embryo transfer has been in progress for a long time in order to produce excellent performance and transgenic offspring in cattle. For the successful production of offspring through embryo transfer to a surrogate mother, the production of a high-quality blastocyst is more important than anything else because embryos at the blastocyst stage is transferred (Choe et al., 2010). In addition, *in vitro* matured and fertilized oocytes are used to produce embryos used for embryo transfer. However, in the case of transgenic embryos produced using *in vitro* matured oocytes, the productivity of transgenic embryos was lower than that of normal *in vitro* fertilized embryos, and due to this problem, the pregnancy rate and production rate of offspring through embryo transfer were lower than those of normal *in vitro* fertilized embryos (Uhm et al., 2013). In order to solve this problem, many researchers have been developing an *in vitro* culture system for a long time to produce high-quality blastocysts using *in vitro* fertilized embryos (Larson et al., 1992; Voelkel and Hu, 1992; Abe and Hoshi, 2003; Lima et al., 2006; Lee et al., 2011; Arat et al., 2016; Ramos-Deus et al., 2020). Therefore, this study was conducted to enhance the embryonic development of transgenic embryos to blastocyst stage.

As shown in Table 1, SCM was used as an additive to CR1aa medium to enhance the embryonic development of *in vitro* fertilized zygotes. The *in vitro* fertilized zygotes were cultured in each CR1aa medium supplemented with 0.3% BSA, 10% FBS or 10% SCM, respectively. As a result, in the embryonic development of *in vitro* fertilized zy-

gotes, the blastocyst and hatching rates of fertilized zygotes in CR1aa medium supplemented with SCM showed significantly higher ($p < 0.05$) than those in each CR1aa medium supplemented with BSA or FBS. Also, the embryonic development of cleaved embryos on day 3 after fertilization, the blastocyst and hatching rates of fertilized oocytes in CR1aa medium supplemented with SCM showed significantly higher ($p < 0.05$) than those in each CR1aa medium supplemented with BSA or FBS (Table 2). Even by other researchers, in mice, the supplement of ESC conditioned medium in culture medium for *in vitro* maturation of oocytes were increased the development to blastocyst stage after fertilization, and these blastocysts were gave birth to normal healthy offspring after transplantation into surrogate mothers (Miraki et al., 2017). In pig, Kim and Park (2019) reported ESC conditioned medium mouse ESC conditioned medium was affected embryonic development ability after fertilization as well as the improvement for maturation by supplement of ESC conditioned medium mouse ESC conditioned medium during *in vitro* maturation oocytes. Also, in the study of human endothelial progenitor cells (EPCs), the culture medium of EPCs secrete numerous growth factors and cytokines such as bFGF, VEGF, IGF-1, IL-10 and EGF. The addition of culture medium of EPCs during *in vitro* maturation of pig oocytes was increased the development to blastocyst stage (Lee, 2020). However, many growth-related substances are present in the ESC conditioned medium (Bendall et al., 2009), and these substances are considered to be helpful in enhancing embryo development. Especially, since the proliferation of many cells is required for late embryonic development for blastocysts and hatching after embryonic genomic activation, many growth-related substances present in ESC conditioned medium are expected to help promote late embryonic development.

Furthermore, in the study for the enhancement of embryonic development of transgenic embryos, the fertilized zygotes at 18 h after *in vitro* fertilization were microinjected of medium into cytoplasm or pronucleus. In the case, these cytoplasmic and pronucleus microinjected cleaved embryos of medium on the day 3 after fertilization were cultured in each CR1aa medium with FBS or SCM. The blastocyst and hatching rates of these all microinjected cleaved embryos of medium in CR1aa medium supplemented with SCM showed significantly higher ($p < 0.05$) than those in CR1aa medium supplemented with FBS (Table 3 and 4). As a traditional method of introducing foreign genes into eggs, microinjection into the pronucleus of fertilized zygotes was used. However, the efficiency of the microinjection method into the pronucleus of fertilized zygotes has been questioned due to the low production of transgenic offspring due to breeding gains. For this reason, the vector carrying the foreign gene was randomly inserted into the genome by linking a general promoter with the gene to be expressed. The production efficiency of transgenic offspring by this method is very low, and the position of the inserted gene is also randomly inserted, so it is common that promoter-specific gene expression is not expressed or its expression is low (Robl et al., 2007). To solve this problem, genetic scissors such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas systems have recently been developed to produce transgenic animals more easily than the classical production method of transgenic animals (Petersen, 2017). The gene editing system is one of the techniques for producing transgenic embryos, and they produced transgenic offspring by injecting genes into the cytoplasm of fertilized eggs (Proudfoot et al., 2015; Wang et al., 2015; Park et al., 2017; Wei et al., 2018). Therefore, it is essential to improve the quality of blastocysts in order to more successfully produce transgenic offspring by microinjecting foreign genes into the cytoplasm of fertilized eggs using such a gene editing system in cattle.

CONCLUSION

This study confirmed that the supplement of SCM in the culture medium for the development of bovine *in vitro* fertilized embryos was enhanced the development of blastocyst and hatching stages. In addition, even when microinjected of medium into the cytoplasm and nucleus of fertilized zygotes, it was investigated that the supplement of SCM in the culture medium was enhanced the development of blastocyst and hatching stages. Therefore, this study is considered that it will help improve the quality of blastocysts derived from *in vitro* fertilized and transgenic embryos for the production of superior and transgenic offspring through embryo transfer in cattle in the future. Especially, these results suggest that the addition of SCM to the *in vitro* culture medium of transgenic embryos will

help solve the problems of low pregnancy rate and offspring production in transgenic animals by improving the quality of blastocysts.

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