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# Effect of bovine oviductal epithelial cell lysate on the developmental competence and quality of bovine *in vitro* fertilized embryos

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ABSTRACT

*In vitro* fertilization (IVF) technology for embryo production has been applied in basic research, animal husbandry and medicine. However, the developmental efficiency and quality of embryos produced by IVF are inferior to those produced *in vivo*. In this study, we investigated the effects of supplementing bovine oviductal epithelial cells (BOEC) lysate during the *in vitro* culture period on the developmental competence and quality of bovine embryos. IVF embryos were cultured for 4 days post-IVF in medium supplemented with 10 % BOEC lysate at various concentrations ($1.0 \times 10^5$, $2.0 \times 10^5$, and $4.0 \times 10^5$ cells/mL) or 10 % PBS (−), which was used to adjust the lysate concentration (control). BOEC lysate at $2.0 \times 10^5$ cells/mL significantly increased the blastocyst formation rate compared to that in the control group. Blastocysts from BOEC lysate supplemented groups showed significantly lower apoptosis rate than that in the control group. The ratio of inner cell mass cell number in blastocysts was significantly higher in all BOEC lysate supplemented groups than in the control group. The survival rate after vitrification/thawing was improved in the $1.0 \times 10^5$ and $2.0 \times 10^5$ cells/mL BOEC lysate supplemented groups. In addition, gene expression analysis of blastocysts showed that $2.0 \times 10^5$ cells/mL of BOEC lysate supplementation significantly enhanced the expression of anti-apoptotic genes (*BCL2* and *BIRC5*), antioxidant-related genes (*GPX1* and *SOD2*), and cell differentiation-related genes (*SOX2* and *OCT4*). In conclusion, supplementation with $2.0 \times 10^5$ cells/mL BOEC lysate during early *in vitro* culture improved the developmental competence and quality of bovine IVF embryos.

## 1. Introduction

*In vitro* fertilization (IVF) enhances our understanding of fertilization and development, while also contributing to the production of high-quality livestock, with increasing demand due to the global adoption of the Ovum Pick Up (OPU) technique [1]. However, IVF embryos have lower developmental efficiency, quality, and cryotolerance compared to *in vivo*-produced embryos, with reduced conception rate post-embryo transfer (ET) [2–4]. These quality differences are due to the differences between *in vivo* and *in vitro* culture, particularly the lack of natural oviductal fluid components *in vitro* culture medium [5,6]. Various approaches have been investigated to improve *in vitro* culture conditions, including the addition serum [7,8], growth factors [9,10], and co-culturing embryos with somatic cells, particularly oviductal epithelial cells (OEC) [11].

The oviduct provides an optimal environment for fertilization and early embryogenesis [12]. In bovine, the oviduct is the site of embryonic development for approximately 4 days after fertilization [13]; the OEC secretes metabolic substrates such as pyruvate, amino acids, and glucose [14,15], as well as various growth factors, such as insulin-like growth

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factor 1 (IGF1) and epidermal growth factor (EGF) [16,17] and is responsible helping the embryo eliminate stress and toxins [18]. Co-culture of IVF embryos with OEC has been studied in various mammalian species, including mouse, porcine, and bovine [11,19,20]. Bovine oviductal epithelial cells (BOEC) are utilized for co-culture and conditioned medium, and have shown beneficial effects in improving the potential and quality of embryo development [21–24]. However, co-culture is labor-intensive, and cell quality may be affected by culture duration and passages [25].

Recently, several cell lysis methods have been introduced [26], and applications of cell lysates in regenerative medicine have been reported for a variety of cells. For example, platelet lysate assists in cell growth and wound healing therapy [27]. In addition, cell lysates have been used to study the expression, modification, and interactions of biomolecules, including proteins and nucleic acids [26,28]. Manabe et al. showed that the addition of adipose stem cell-derived lysate to the culture process of bovine IVF embryos improved embryonic development and cryotolerance [29]. Therefore, if the addition of BOEC lysate improves the developmental potential of IVF embryos, this approach could serve as a more convenient alternative to the conventional co-culture method. The objective of this study was to evaluate the effectiveness of the supplementing of BOEC lysate in the production of bovine IVF, using the 4-day period during which the *in vivo* embryos remain in the oviduct as a baseline.

## 2. Materials and methods

### 2.1. Chemicals

Unless otherwise specified, all chemicals and reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). All experiments and procedures were conducted in accordance with the Guidelines for Animal Research and Welfare of Gifu University Animal Research Ethics Committee.

### 2.2. Establishment of bovine oviductal epithelial cells (BOEC)

Oviducts were collected from a Japanese Black cattle at a local slaughterhouse and transported to the laboratory in 0.85 % saline with 100 μg/mL kanamycin sulfate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at 30–33 °C within 2 h. They were flushed with PBS (−) containing 2 % Penicillin-Streptomycin Solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and opened longitudinally, and the epithelial tissue was scraped using a sterile spatula. The tissue was washed twice by centrifugation at 600×*g* for 5 min, resuspended in cell culture medium (D-MEM/Ham's F-12 with L-glutamine and phenol red [Wako] supplemented with 20 % FBS [Gibco, Grand Island, NY, USA], 2 % penicillin-streptomycin and 0.5 % amphotericin B, and cultured in a 75 $cm^2$ flask (PT. IWAKI Glass Indonesia, West Java, Indonesia) at 37 °C in 5 % $CO_2$. After reaching confluence, cells were washed with PBS (−), treated using 0.25 % (w/v) trypsin/1 mmol/L EDTA・4Na solution with phenol red (Wako) for 5 min, and the reaction was stopped using PBS (−) with 10 % FBS. Cells were centrifuged at 200×*g* for 5 min and cultured through three passages, reducing the FBS concentration to 10 % after second passage.

### 2.3. Preparation of BOEC lysate

Confluent BOEC were washed with PBS (−), incubated for 5 min with 0.25 % (w/v) trypsin/1 mmol/L EDTA・4Na solution to disperse them from the flask and trypsin activity was stopped using PBS (−) with 10 % FBS. The cells were collected in a 50 mL tube, centrifuged at 200×*g* for 5 min, washed with PBS (−), and centrifuged again in the same conditions. The pellet was resuspended in PBS (−), and cells count was determined using a Thoma hemocytometer. The cell suspension was frozen at −25 °C overnight to lysate the cells. On the following day, it was thawed at room temperature, centrifuged (800×*g*, 3 min), filtrated using a 0.2 μm membrane filter, and stored at −25 °C until use. Experiments utilized BOEC from passage 4–5.

### 2.4. Oocyte collection and in vitro maturation (IVM)

Japanese Black cattle ovaries were collected at a local slaughterhouse and transported to the laboratory at 33–34 °C in 0.85 % saline supplemented with 100 μg/mL kanamycin sulfate within 3 h. Immature cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm follicles using an 18-gauge needle attached to a 5 mL disposable syringe. The follicular fluid was placed in a 15 mL conical tube, and the sediment containing COCs was transferred to a 90 mm dish with Hepes-buffered Tyrode-lactate-pyruvate-polyvinyl alcohol (Hepes-TLP-PVA). COCs of high quality were selected and cultured in a maturation medium (TCM199 with Earle's salts [Gibco, BRL, Grand Island, NY, USA] and 10 % FBS) in a humidified atmosphere of 5 % $CO_2$ at 38.5 °C for 22 h.

### 2.5. Sperm preparation and in vitro fertilization (IVF)

IVF was performed using BO-theophylline (BO-medium [30] and 10 mM theophylline) and BO-BSA-heparin (BO-medium, 1 % BSA, and 2.5 IU/mL heparin [Novo Nordisk Pharma Ltd., Tokyo, Japan]). Frozen semen from a Japanese Black bull was thawed at 38.0 °C for 20 s and washed twice by centrifugation at 600×*g* for 5 min in 8 mL of BO-theophylline. Sperm concentration was adjusted to $1.5 \times 10^7$ sperms/mL using BO-theophylline and BO-BSA-heparin. COCs after 22 h of IVM were partly denuded of cumulus cells by gentle pipetting with Hepes-TLP-PVA containing 0.25 % (w/v) hyaluronidase. Groups of 20–30 COCs were transferred to sperm droplets and co-incubated for 6 h at 38.5 °C in a humidified atmosphere of 5 % $CO_2$.

### 2.6. In vitro culture (IVC) and assessment of embryonic development

CR1aa medium [31] was used as the basal medium for IVC. After co-culture for 6 h, cumulus cells and sperm attached to the zona pellucida were completely removed from the presumptive zygotes by mechanical pipetting with Hepes-TLP-PVA containing 0.1 % hyaluronidase. The presumptive zygotes were then transferred to CR1aa medium supplemented with 0.3 % BSA and various concentrations of BOEC lysate. Different concentrations of BOEC lysate were prepared by dilution with PBS (−). It was then added to the IVC medium at a rate of 10 % to achieve a final concentration of $1.0 \times 10^5$, $2.0 \times 10^5$, or $4.0 \times 10^5$ cells/mL. PBS (−) used for dilution was added to CR1aa medium at 10 % to make a control group. The presumptive zygotes were incubated for 4 days at 38.5 °C in a humidified atmosphere of 5 % $O_2$, 5 % $CO_2$, and 90 % $N_2$. They were then transferred to CR1aa medium with 10 % FBS and incubated for another 3 days. The day of IVF was used as day 0 of culture, cleavage rate was observed on day 2, and blastocyst formation rate was observed on day 7 using an inverted microscope (IX70; Olympus, Tokyo, Japan).

### 2.7. Assessment of apoptosis and total cell number in blastocyst

Apoptosis and total cell number in blastocysts were analyzed using a TUNEL assay with *In Situ* Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, IN, USA) and Hoechst 33342 staining (Dojindo Laboratories, Kumamoto, Japan). Blastocysts were fixed with 4 % paraformaldehyde at room temperature for 30 min, permeabilized with 0.5 % (w/v) Triton X-100 at room temperature for 30 min. They were incubated with the TUNEL assay reagent at 38.5 °C for 1 h in the dark, then washed twice with PVA-PBS [0.3 % polyvinyl alcohol in PBS (−)]. The blastocysts were stained with 10 μg/mL Hoechst 33342 for 15 min at 4 °C, mounted on glass slides, and image using a fluorescence microscope (BH-2; Olympus) equipped with a digital camera. Apoptotic and total cell numbers were counted using ImageJ software (version

1.41; National Institutes of Health, Bethesda, MD, USA).

### 2.8. Assessment of ICM and TE in blastocyst

ICM and TE in blastocysts were assessed by immunostaining and Hoechst 33342 staining. Blastocysts were fixed with 4 % paraformaldehyde for 30 min, permeabilized with 1 % (w/v) Triton X-100, and blocked with PBS (−) containing 0.5 % BSA and 10 % horse serum (Gibco) at 4 °C for 2 h. They were incubated overnight at 4 °C with a rabbit anti-CDX2 monoclonal antibody (1:50; Cell Signaling Technology, Danvers, MA, USA, #12306), followed by incubation with anti-rabbit IgG (H + L), F (ab') fragment (1:500; Cell Signaling Technology, #4413) for 1 h. Blastocysts were then stained with 10 μg/mL Hoechst 33342 for 15 min, mounted on glass slides, and imaged using a fluorescence microscope with a digital camera. The numbers of TE and total cells were counted using ImageJ software, and the ICM cell count was calculated by subtracting TE cells from the total cell count.

### 2.9. Vitrification and warming

Blastocysts were vitrified using a Cryotop® device (KITAZATO Co., Shizuoka, Japan). They were immersed in equilibrium solution (TCM199 with 7.5 % ethylene glycol, 7.5 % dimethyl sulfoxide, 20 % FBS and 0.1 % gentamycin) for 12–15 min, followed by vitrification solution (TCM199 with 15 % ethylene glycol, 15 % dimethyl sulfoxide, 20 % FBS, 0.2 % gentamycin, and 10 % sucrose) for 60–70 s. The blastocysts were then placed on the Cryotop® and immediately immersed in liquid nitrogen. Thawing was performed by immersing the Cryotop® in a thawing solution (TCM199 with 20 % FBS, 6.5 % sucrose, and 0.03 % gentamycin) at 38.5 °C, followed by transfer to a culture medium (TCM199, 10 % FBS and 1 % β-mercaptoethanol). Blastocysts were cultured for 72 h at 38.5 °C in 5 % $CO_2$, and survival was assessed by intact cytoplasm and re-expansion at 24, 48, and 72 h. In addition, the hatching rate was also evaluated.

### 2.10. Total RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from 10 blastocysts per group (control and $2.0 \times 10^5$ cells/mL BOEC lysate group) using a direct method as previously described [32]. Briefly, blastocysts were washed with PVA-PBS, followed by wash in freezing solution (DEPC treatment water, 0.15 M NaCl and 10 mM Tris), then mixed with RNase Inhibitor stock (freezing solution, 5 mM dithiothreitol and 10 % RNase Inhibitor). Samples were immersed in liquid nitrogen. cDNA was synthesized using the QuantAccuracy®, RT-RamDA® cDNA Synthesis Kit (TOYOBO, Osaka, Japan) according to the instructions provided by manufacturer. Reactions included denaturation, priming, reverse transcription, and amplification steps, performed in a the TaKaRa PCR Thermal Cycler Dice™ Touch (TaKaRa Bio, Shiga, Japan). Real-time PCR was conducted with the Power Track SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) on QuantStudio 3 (Applied Biosystems). The program included pre-incubation (95 °C for 5 min), followed by 40 cycles of denaturation (95 °C for 10 s), annealing (55 °C for 15 s), and extension (72 °C for 30 s). Relative expression levels were calculated using ΔΔCt method, normalized to housekeeping gene (*β-actin*). Primers used are listed in Table 1.

### 2.11. Experimental design

In this study, the following four experiments were conducted.

**Experiment 1**. Effect of supplementing various concentrations of BOEC lysate on embryo developmental competence.

Zygotes were cultured for 4 days in medium with 10 % BOEC lysate ($1.0 \times 10^5$, $2.0 \times 10^5$, and $4.0 \times 10^5$ cells/mL) or 10 % PBS (−) as a control. The cleavage rate (day 2) and the blastocyst formation rate (day 7) were assessed.

**Experiment 2**. Effect of supplementing various concentrations of BOEC lysate on embryo quality.

Zygotes were cultured under the same conditions as experiment 1, blastocysts (day 7) were analyzed for total cell number, apoptotic cell rate, ICM cell rate, and TE cell rate.

**Experiment 3**. Effect of supplementing various concentrations of BOEC lysate on embryo cryotolerance.

Zygotes were cultured under the same conditions as in experiment 1, blastocysts (day 7) were vitrified and then thawed. Survival rates were assessed at 24, 48, and 72 h after thawing. Hatching rates were also measured.

**Experiment 4**. Effect of supplementing BOEC lysate on gene expression in blastocyst.

Zygotes were cultured in a medium with 10 % BOEC lysate ($2.0 \times 10^5$ cells/mL) or 10 % PBS (−) for 4 days, followed by 3 days in lysate-free medium. Gene expression profiles of blastocysts obtained on day 7

**Table 1**
Details of primers used for reverse transcription-quantitative polymerase chain reaction.

| Gene | Primer sequence (5′-3′) | | Fragment size (bp) | Accession No. |
|---|---|---|---|---|
| *BAX* | Forward: | TCTGACGGCAACTTCAACTG | 135bp | NM_173894 |
| | Reverse: | TCGAAGGAAGTCCAATGTCC | | |
| *BCL2* | Forward: | GTCAACCGGGAGATGTCG | 163bp | NM_001166486.1 |
| | Reverse: | GACAGCCAGGAGAAATCAAACA | | |
| *BIRC5* | Forward: | CCTGGCAGCTCTACCTCAAG | 233bp | AY606044 |
| | Reverse: | GAAAGCACAACCGGATGAAT | | |
| *GPX1* | Forward: | GGACTACACCCAGATGAATGAC | 132bp | NM_174076.3 |
| | Reverse: | TACTTCAGGCAATTCAGGATCTC | | |
| *SOD2* | Forward: | GACGCTTACAGATTGCTGCTTGT | 127bp | NM_201527.2 |
| | Reverse: | TCGGGCCTGACATTTTTATACTG | | |
| *SOX2* | Forward: | ATGATGGAGACGGAGCTGAA | 113bp | NM_001105463 |
| | Reverse: | GGGCTGTTCTTCTGGTTGC | | |
| *CDX2* | Forward: | GCCACCATGTACGTGAGCTAC | 140bp | NM_001206299 |
| | Reverse: | ACATGGTATCCGCCGTAGTC | | |
| *OCT4* | Forward: | AGGTGTTCAGCCAAACGACTAT | 145bp | NM_174580 |
| | Reverse: | GTCTCTGCCTTGCATATCTCCT | | |
| *GLUT1* | Forward: | CAGGAGATGAAGGAGGAGAGC | 258bp | M60448.1 |
| | Reverse: | CACAAATAGCGACACGACAGT | | |
| *β-actin* | Forward: | CTCTTCCAGCCTTCCTTCCT | 178bp | NM_173979.3 |
| | Reverse: | GGGCAGTGATCTCTTTCTGC | | |

were analyzed.

### 2.12. Statistical analysis

All percentage data (cleavage, blastocyst formation, and survival rates after vitrification) were subjected to arcsine transformation before analysis. All data, except gene expression data, were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test. Gene expression was compared using Student's *t*-test. All values are presented as the mean ± standard error of the mean (SEM), and statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Effect of supplementing various concentrations of BOEC lysate on embryo developmental competence

The cleavage rate was significantly higher ($P < 0.05$) in the $1.0 \times 10^5$ cells/mL BOEC lysate supplemented group than that in the control and $4.0 \times 10^5$ cells/mL BOEC lysate supplemented groups (Table 2). In addition, the rate of cleaved ≥4 cell embryos were the highest in the $1.0 \times 10^5$ cells/mL BOEC lysate supplemented group and was significantly higher ($P < 0.05$) than that in the $4.0 \times 10^5$ cells/mL BOEC lysate supplemented group. In contrast, the blastocyst formation rate was significantly higher ($P < 0.05$) in the $2.0 \times 10^5$ cells/mL BOEC lysate supplemented group than that in the control group (Table 2). Moreover, morphologically normal blastocysts were obtained in all experimental groups (Supplementary Fig. 1).

### 3.2. Effect of supplementing various concentrations of BOEC lysate on embryo quality

As shown in Table 3, the total number of cells in blastocysts did not differ between the experimental groups. In contrast, the number and rate of apoptotic cells were significantly lower ($P < 0.05$) in all BOEC lysate supplemented groups than that in the control. Fluorescence images of each experimental group are shown in Supplementary Fig. 2. As shown in Table 4, the number of cells in the ICM was significantly higher ($P < 0.05$) in the $1.0 \times 10^5$ and $2.0 \times 10^5$ cells/mL BOEC lysate supplemented groups than that in the control. In addition, the ICM cell ratio was significantly higher ($P < 0.05$) in all BOEC lysate supplemented groups than that in the control, while the TE cell ratio was significantly lower ($P < 0.05$) in all BOEC lysate supplemented groups. Fluorescence images of each experimental group are shown in Supplementary Fig. 3.

**Table 2**
Effect of various concentrations of BOEC lysate supplementation during the first 4 days of IVC on *in vitro* developmental capacity of bovine *in vitro* fertilized embryos.

| BOEC lysate concentration | Number of embryos examined | No. (% ± SEM)[a] of embryos developed to | | | | |
|---|---|---|---|---|---|---|
| | | Day 2 | | | | Day 7 |
| | | 2 cell | 3 cell | 4 cell ≦ | cleavage | blastocyst |
| PBS (−) | 111 | 5 (4.5 ± 1.7) | 1 (0.9 ± 0.8) | 53 (47.7 ± 3.6)[ab] | 59 (53.2 ± 3.4)[b] | 32 (28.8 ± 1.2)[b] |
| $1.0 \times 10^5$ cells/mL | 111 | 9 (8.1 ± 2.6) | 4 (3.6 ± 1.5) | 67 (60.4 ± 4.9)[a] | 80 (72.1 ± 3.3)[a] | 38 (34.2 ± 2.2)[ab] |
| $2.0 \times 10^5$ cells/mL | 113 | 5 (4.4 ± 0.3) | 5 (4.4 ± 1.7) | 60 (53.1 ± 3.4)[ab] | 70 (61.9 ± 4.0)[ab] | 42 (37.2 ± 1.0)[a] |
| $4.0 \times 10^5$ cells/mL | 111 | 7 (6.3 ± 1.2) | 6 (5.4 ± 3.4) | 49 (44.1 ± 2.4)[b] | 62 (55.9 ± 2.2)[b] | 36 (32.4 ± 2.3)[ab] |

[a-b] Values with different superscripts within the same column are significantly different ($P < 0.05$).
The experiment was repeated five times.
[a] Percentage of total cultured embryos.

**Table 3**
Effect of various concentrations of BOEC lysate supplementation during the first 4 days of IVC on the total cell number and apoptotic cell number of bovine *in vitro* fertilized embryos.

| BOEC lysate concentration | Number of embryos examined | Total cell number | Apoptotic cell number | Apoptotic rate** (% ± SEM) |
|---|---|---|---|---|
| PBS (−) | 32 | 141.7 ± 6.5 | 8.7 ± 1.0[b] | 6.1 ± 0.7[b] |
| $1.0 \times 10^5$ cells/mL | 38 | 155.5 ± 7.4 | 5.9 ± 0.7[a] | 3.9 ± 0.4[a] |
| $2.0 \times 10^5$ cells/mL | 42 | 147.9 ± 7.6 | 5.2 ± 0.5[a] | 3.7 ± 0.3[a] |
| $4.0 \times 10^5$ cells/mL | 36 | 129.9 ± 5.6 | 5.4 ± 0.6[a] | 4.2 ± 0.5[a] |

Data are mean ± SEM Values.
[a-b] Values with different superscripts within the same column are significantly different ($P < 0.05$).
The experiments were replicated five times.
*Percentage of apoptotic cell number to total cell number.

### 3.3. Effect of supplementing various concentrations of BOEC lysate on embryo cryotolerance

The survival rates at 24, 48, and 72 h post-vitrification/thawing were significantly improved ($P < 0.05$) in the $1.0 \times 10^5$ and $2.0 \times 10^5$ cells/mL BOEC lysate supplemented groups compared to that in the control (Table 5 and Fig. 1). In contrast, the $4.0 \times 10^5$ cells/mL BOEC lysate supplemented group did not show a significant difference compared to the other experimental groups. The hatching rates at 48 and 72 h post-vitrification/thawing showed a tendency to improve in the BOEC lysate supplemented groups compared to the control.

### 3.4. Effect of BOEC lysate on the gene expression in blastocyst

As shown in Fig. 2, no differences were observed in the expression of apoptotic genes (*BAX*), cell differentiation-related gene (*CDX2*), or glucose transporter-related gene (*GLUT1*). In contrast, the expressions of anti-apoptotic genes (*BCL2* and *BIRC5*), antioxidant-related genes (*GPX1* and *SOD2*), and cell differentiation-related genes (*SOX2* and *OCT4*) were significantly higher ($P < 0.05$) in the $2.0 \times 10^5$ cells/mL BOEC lysate supplemented group than that in the control group.

## 4. Discussion

This study evaluated the efficacy of supplementing IVC medium with BOEC lysate in bovine IVF embryo production. In our preliminary experiments, the addition of BOEC lysate during the entire 7 days of the IVC period did not improve developmental competence and, on the contrary, delayed development (data not shown). However, we deduced from the results that the effect could not be obtained unless the lysate was added during the period of stay in the oviduct and that the BOEC lysate contains bioactive substances that affect the developmental competence. Therefore, we set the supplementation period of BOEC lysate at 4 days, which is the period during which embryos normally remain in the oviduct *in vivo*. The results of this study indicate that supplementation of the medium with $2.0 \times 10^5$ cells/mL BOEC lysate for 4 days after fertilization improves the developmental competence, quality and gene expression profile of bovine IVF embryos.

Early embryonic development is one of the most important and complex stages, with the post-fertilization environment influencing the

**Table 4**
Effect of various concentrations of BOEC lysate supplementation during the first 4 days of IVC on the cell number of inner cell mass (ICM) and trophectoderm (TE) cell of bovine *in vitro* fertilized embryos.

| BOEC lysate concentration | Number of embryos examined | Total cell number | ICM cell number | TE cell number | ICM cell rate[a] (% ± SEM) | TE cell rate*** (% ± SEM) |
|---|---|---|---|---|---|---|
| PBS (−) | 27 | 133.7 ± 7.6 | 41.3 ± 2.7$^{c}$ | 92.3 ± 5.3 | 30.7 ± 0.9$^{b}$ | 69.3 ± 0.9$^{a}$ |
| 1.0 × $10^5$ cells/mL | 28 | 146.0 ± 7.6 | 56.9 ± 3.5$^{ab}$ | 89.1 ± 4.5 | 38.6 ± 0.9$^{a}$ | 61.4 ± 0.9$^{b}$ |
| 2.0 × $10^5$ cells/mL | 34 | 153.8 ± 6.7 | 62.2 ± 2.9$^{a}$ | 91.6 ± 4.3 | 40.7 ± 0.9$^{a}$ | 59.3 ± 0.9$^{b}$ |
| 4.0 × $10^5$ cells/mL | 24 | 133.3 ± 8.1 | 49.4 ± 3.2$^{bc}$ | 83.9 ± 5.7 | 37.5 ± 1.4$^{a}$ | 62.5 ± 1.4$^{b}$ |

Data are mean ± SEM Values.
[a-c] Values with different superscripts within the same column are significantly different ($P < 0.05$).
The experiments were replicated seven times.
*Percentage of ICM cell number to total cell number.
[a] Percentage of TE cell number to total cell number.

**Table 5**
Effect of various concentrations of BOEC lysate supplementation during the first 4 days of IVC on the survival and hatching rate of bovine *in vitro* fertilized embryos after vitrification/thawing.

| BOEC lysate concentration | Number of embryos examined | No. (% ± SEM)[a] of embryos after vitrification/thawing to | | | | | |
|---|---|---|---|---|---|---|---|
| | | 24 h | | 48 h | | 72 h | |
| | | Survival | Hatched | Survival | Hatched | Survival | Hatched |
| PBS (−) | 13 | 7 (53.8 ± 6.9)$^{b}$ | 0 (0.0 ± 0.0) | 5 (38.5 ± 4.1)$^{b}$ | 1 (7.7 ± 9.1) | 4 (30.8 ± 6.0)$^{b}$ | 1 (7.7 ± 9.1) |
| 1.0 × $10^5$ cells/mL | 13 | 12 (92.3 ± 9.1)$^{a}$ | 0 (0.0 ± 0.0) | 10 (76.9 ± 8.5)$^{a}$ | 4 (30.8 ± 15.7) | 9 (69.2 ± 1.3)$^{a}$ | 6 (46.2 ± 10.4) |
| 2.0 × $10^5$ cells/mL | 14 | 14 (100.0 ± 0.0)$^{a}$ | 0 (0.0 ± 0.0) | 10 (71.4 ± 9.7)$^{a}$ | 4 (28.6 ± 12.9) | 9 (64.3 ± 1.1)$^{a}$ | 5 (35.7 ± 14.7) |
| 4.0 × $10^5$ cells/mL | 18 | 13 (72.2 ± 10.7)$^{ab}$ | 0 (0.0 ± 0.0) | 11 (61.1 ± 16.5)$^{ab}$ | 4 (22.2 ± 12.7) | 10 (55.5 ± 15.8)$^{ab}$ | 6 (33.3 ± 23.7) |

[a-b] Values with different superscripts within the same column are significantly different ($P < 0.05$).
The experiments were replicated three times.
[a] Percentage of the number of vitrification-thawing embryos.

developmental potential and quality of the embryo [33]. Some differences exist between *in vivo* and *in vitro* culture environments, and the *in vitro* culture medium lack growth factors, hormones, and other components found in oviductal fluid [5,6]. The addition of oviductal fluid [25,34], co-culture with BOEC [11,21,22], and conditioned medium [23,24] improve the developmental competence and quality of bovine IVP embryos. In this study, we supplemented the culture medium of bovine IVF embryos with BOEC lysate as a simpler and more efficient method than conventional techniques.

In experiment 1, it was found that the supplementation of the embryo culture medium with BOEC lysate for 4 days enhanced the developmental competence of bovine IVF embryos (Table 2). Pyruvate, lactate, and amino acids are important trophic factors involved in early mammalian embryonic development [35]. Pyruvate is a major energy source for embryonic development up to the 8–16 cell stage [36]. It has been reported that co-culturing bovine IVF embryos with BOEC during the first 48 h of development increases the concentration of pyruvate and lactate in the medium [37] and enhances the developmental capacity of bovine embryos [38,39]. Therefore, pyruvate and lactate in the culture medium were possibly increased by supplementation with BOEC lysate, and the increased pyruvate metabolic activity improved developmental competence, which resulted in an increased cleavage rate (Table 2).

In this study, the blastocyst formation rate was significantly higher ($P < 0.05$) in the 2.0 × $10^5$ cells/mL BOEC lysate supplemented group than that in the control group (Table 2). The oviduct contains trophic factors such as amino acids, carboxylic acid, and glucose, as well as growth factors such as IGF1 and transforming growth factor β (TGFβ), which have a significant effect on embryonic development *in vivo* [40–42]. In bovine embryos, metabolism gradually switches to glucose as a substrate between the 8–16 cell stage as more energy is required for compaction and subsequent blastocyst formation [43,44]. In addition, IGF1 secreted by BOEC increases the uptake of amino acids and glucose in the embryo and improves the developmental competence of bovine IVF embryos [45]. Therefore, the supply of glucose and IGF1 to the medium by 2.0 × $10^5$ cells/mL BOEC lysate may have stimulated glucose metabolism, further promoting embryonic development and resulting in a high blastocyst formation rate.

In experiment 2, the effect of BOEC lysate on embryo quality was evaluated by examining apoptosis, ICM cell rate, and TE cell rate in blastocysts. The number and rate of apoptotic cells were significantly lower ($P < 0.05$) in all BOEC lysate supplemented groups than that in the control group (Table 3). Apoptosis is a cell death process that is intricately programmed by apoptosis-promoting and -suppressing factors, allowing embryo survival through the removal of damaged cells [46,47]. In the *in vitro* culture environment, light and temperature changes and embryo manipulation induce apoptosis by producing excess reactive oxygen species (ROS) and reducing the antioxidant functions of the embryo [48,49]. In contrast, BOEC secretes antioxidant enzymes such as glutathione peroxidase and catalase [50,51]. It has been reported that co-culturing embryos with BOEC or adding oviductal fluid to the medium increases the expression of antioxidant-related genes such as *SOD2*, *GPX1*, and *GPX4*, and decreases ROS levels in bovine IVF embryos [11,34,52]. IGF1 also functions as an apoptosis inhibitor and its addition to the culture medium reduces apoptosis in mouse and bovine IVF embryos [53]. Furthermore, the expression of the anti-apoptotic gene *BIRC5* in bovine embryos is increased by IGF1 and EGF treatment [54]. In experiment 4, a significant increase in the expression of antioxidant-related genes (*SOD2* and *GPX1*) and anti-apoptotic genes (*BCL2* and *BIRC5*) was observed upon supplementation of 2.0 × $10^5$ cells/mL BOEC lysate (Fig. 2). Therefore, the growth factors in BOEC lysate may have contributed to the reduction of ROS and apoptosis in embryos by improving the expression of anti-apoptotic and antioxidant-related genes.

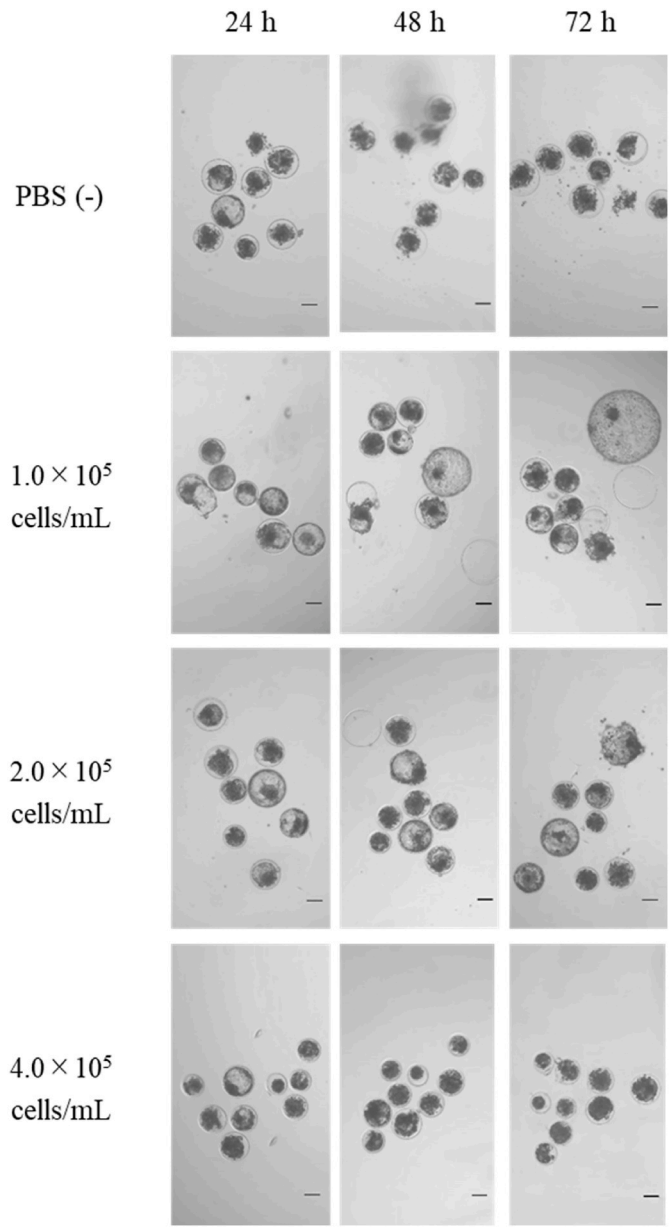


**Fig. 1.** Photographs of blastocysts 24, 48, and 72 h after vitrification/thawing in blastocysts obtained after supplementation with various concentrations of BOEC lysate on the first 4 days of *in vitro* culture (IVC). Scale bar = 100 μm.

Next, the effects of ICM and TE cells on differentiation were evaluated. As shown in Table 4, the number of ICM cells was significantly higher ($P < 0.05$) in the $1.0 \times 10^5$ and $2.0 \times 10^5$ cells/mL BOEC lysate supplemented groups than in the control. In addition, the ratio of ICM cell number to total cell number was significantly higher ($P < 0.05$) in all BOEC lysate supplemented groups than in the control. The first tissue differentiation in a mammalian embryo occurs during the development from the morula stage to the blastocyst stage, dividing into ICM, which develops into the fetus, and the TE, which develops into the placenta [55,56]. The cell numbers of ICM and TE cells are an indicator of blastocyst quality, and the cell number of ICM are involved in the implantation and development of the fetus after ET [57,58]. The improvement of ICM number in this study means the production of high-quality embryos, which are expected to have a high success rate of ET and subsequent developmental potential. In addition, cell differentiation-related genes such as *SOX2* and *NANOG* are involved in the differentiation of ICM, and *CDX2* is involved in the differentiation of TE in porcine and bovine [59]. The addition of porcine oviduct-derived extracellular vesicles (EVs) or BOEC conditioned medium increases the expression of cell differentiation-related genes such as *NANOG* and *OCT4* in porcine somatic cell nuclear transfer embryos or bovine IVF embryos [60,61]. In addition, porcine oviductal fluid growth differentiation factor 8 (GDF8) improved developmental competence, ICM cell number, and *SOX2* expression in porcine IVF embryos [62]. Furthermore, in experiment 4, a significant increase in *SOX2* and *OCT4* expressions was observed after supplementation with $2.0 \times 10^5$ cells/mL BOEC lysate (Fig. 2). Therefore, it is suggested that the BOEC lysate also contained GDF8, which improved the ICM cell number by increasing *SOX2* expression.

In experiment 3, the survival rates after vitrification/thawing were examined to evaluate the cryotolerance of blastocysts. As shown in Table 5, the survival rates at 24, 48, and 72 h post-vitrification/thawing were significantly higher ($P < 0.05$) in the $1.0 \times 10^5$ and $2.0 \times 10^5$ cells/mL BOEC lysate supplemented groups than in the control. Embryos are sensitive to the cold environment and suffer morphological and functional damage due to degeneration of cell membranes and cytoplasm during freezing [63,64]. The factors that cause vitrification/thawing damage are the high lipid content of the embryo and oxidative stress [65,66]. *In vitro* embryos are more sensitive to freezing and thawing than *in vivo* embryos due to their higher lipid content [64,67]. In previous studies, the addition of bovine oviductal fluid or BOEC conditioned medium improved mitochondrial activity, reduced lipid content, and improved the cryotolerance of blastocysts [60,68]. Oxidative stress is caused by an imbalance between ROS and cellular antioxidant capacity [69]. The addition of bovine oviduct fluid or oviduct-derived EVs reduces ROS levels and oxidative stress in bovine blastocysts [34,60]. In experiment 4, the expression levels of *GPX1* and *SOD2*, which are involved in scavenging ROS and reducing oxidative stress [50,51], were significantly increased (Fig. 2). In particular, the expression of *SOD2* correlates with mitochondrial activity [70]. Furthermore, excess ROS reacts with lipids to form lipid peroxide, which contributes to freezing damage [71]. Therefore, BOEC lysate may improve cryotolerance by reducing the lipid content and oxidative stress through improved mitochondrial function. In addition, the hatching rates showed a tendency to improve in the BOEC lysate supplemented groups (Table 5). The activation of IGF1 and mitochondrial transcription factor A (TFAM) improves embryonic development speed and increases blastocyst cell number [72,73]. In this study, supplementation of IGF1 and improvement of mitochondrial function by BOEC lysate may have promoted blastocyst development and increased hatching.

In experiment 4, the expression of many genes in blastocysts were improved by supplementation with BOEC lysate (Fig. 2). Oviduct-derived EVs contain proteins, mRNA, and miRNAs that are transported across the zona pellucida to the embryo [74,75]. miRNAs then bind to the complementary mRNA sequence in the embryo and regulate gene expression [76]. The miRNA regulation alters the expression of a wide range of genes, involved in apoptosis, mitochondrial function, and cell differentiation, thereby improving embryonic development [77]. *GPX1* and *SOD2* are antioxidant-related genes [50,51], and the expression of *SOD2* is related to embryo quality [25,78]. On the other hand, *OCT4* is involved in ICM and TE differentiation, and *SOX2* is involved in ICM differentiation [59]. *In vivo* embryos have shown higher expressions of *OCT4* and *SOX2* than *in vitro* embryos [79,80]. In addition, high-quality bovine blastocysts have higher levels of *GPX1*, *BCL2,* and *SOX2* than low-quality blastocysts [25,81,82]. Based on the results of this study, blastocysts produced by supplementation with BOEC lysate had improved gene expression (Fig. 2), suggesting that mimicking the environment in the bovine oviduct may have resulted in the production of high-quality blastocysts that resembled the *in vivo* embryo.

In previous studies on co-culture with BOEC [11,52], 4-well dish (NUNC, Roskilde, Denmark: 1.9 $cm^2$/well) were used and co-culture under confluent conditions. In this study, when BOEC were cultured in 75 $cm^2$ flask, the confluent cell density was approximately $1.0 \times 10^7$ cells/mL. When converted to one well of a 4-well dish, the BOEC density

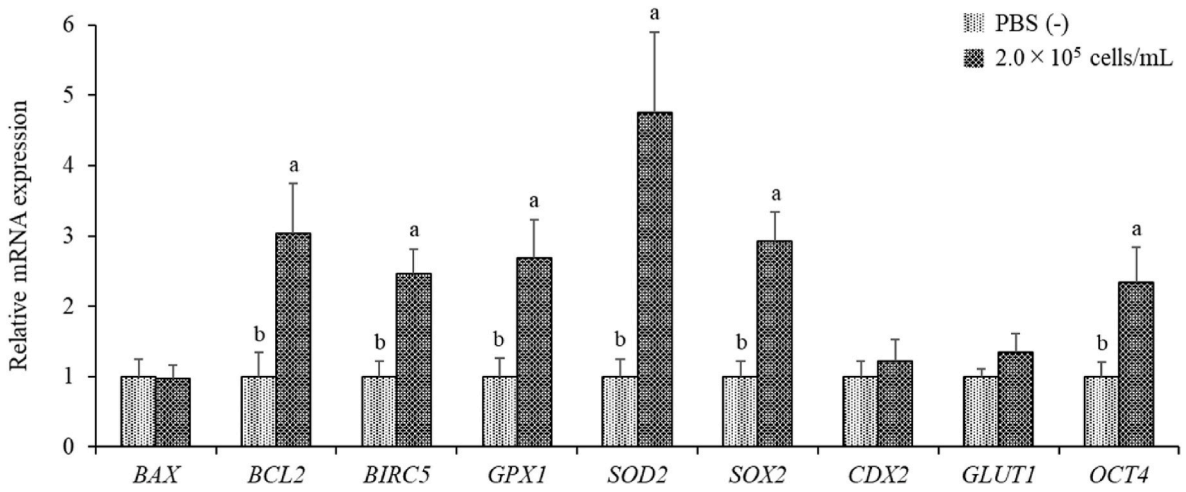


**Fig. 2.** Relative mRNA expression of various genes in blastocysts of the control group and the $2.0 \times 10^5$ cells/mL BOEC lysate supplemented group by real-time PCR. Data are mean ± SEM values. Values with different superscripts for each gene indicate a significant difference ($P < 0.05$).

during co-culture is estimated to be approximately $2.5 \times 10^5$ cells/mL. The optimal concentration of BOEC lysate in this study was $2.0 \times 10^5$ cells/mL, indicating its efficacy at a cell density similar to that used for co-culture. Furthermore, in co-culture and conditioned medium, there is a risk that the amount and composition of bioactive components secreted by the cells may change depending on the culture conditions. On the other hand, lysate is prepared by directly disrupting cells and extracting their contents, which reduces variation in composition and is likely to yield more reproducible results. Thus, the use of lysate is a simpler and more effective method compared to conventional approaches. In this study, BOEC was used without considering the estrous cycle and an improved effect was observed. However, it would be interesting to continuously optimize BOEC characteristics by adding hormones such as luteinizing hormone or estrogens to the BOEC culture medium. In addition, BOEC lysate likely contains nutrient factors such as lactic acid, pyruvate, amino acids, and glucose, as well as growth factors such as IGF1, EGF, TGFβ, and GDF8. It may also contain factors involved in intercellular communication such as EVs and miRNAs. Therefore, it is necessary to identify the components of BOEC lysate. It is also important to verify the effects of BOEC lysate on fertility and calf production after ET.

## 5. Conclusion

In this study, we investigated the effects of BOEC lysate supplementation on the developmental competence and quality of bovine IVF embryos. The results showed that supplementation with $2.0 \times 10^5$ cells/mL BOEC lysate at the early stage of *in vitro* culture improved the developmental competence, quality, cryotolerance, and gene expression profiles in bovine IVF embryos. This study showed that the addition of BOEC lysate produced results similar to those obtained by co-culturing with BOEC, including the addition of conditioning medium, EVs, and oviductal fluid. This new method allows for simpler and more efficient embryo production than conventional methods. These findings will contribute to the advancement of IVP technology and the expansion of ET in livestock production, potentially facilitating more efficient calf production.

## CRediT authorship contribution statement

**Funa Ota:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Hayato Minowa:** Investigation, Formal analysis, Data curation. **Rina Miura:** Investigation, Formal analysis, Data curation. **Tetsuma Murase:** Writing – review & editing, Methodology. **Tokunori Yamamoto:** Writing – review & editing, Funding acquisition. **Takehiro Himaki:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

All authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.02.002.

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