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Lysate of bovine adipose-derived stem cells accelerates in-vitro development and increases cryotolerance through reduced content of lipid in the *in vitro* fertilized embryos

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ABSTRACT

Mesenchymal stem cells such as adipose-derived stem cells (ADSCs) are known to secrete factors that stimulate cell division and promote regeneration in neighboring cells. While conditioned medium from stem cells has been used in blastocyst production, no studies have examined the use of cell lysates. In this study we investigated the effects of adding ADSC lysate to *in vitro* culture (IVC) medium. ADSCs and fibroblasts were isolated from bovine adipose tissue and auricular tissue, respectively, and their lysates were prepared by freeze-thaw disruption. ADSC lysate was added to synthetic oviductal fluid medium. The effects on cleavage, blastocyst development rates, cell numbers, cryotolerance, gene expression (*POU5F1, BAX, IGF1R, IGF2R, SOD2*), lipid content, and membrane integrity were evaluated according to the experimental design. In Expt. 1, the comparison involved adding ADSC or fibroblast lysate alongside the control group. The total blastocyst rate increased when ADSC lysate was introduced (ADSCs: 40.1 %, fibroblasts: 33.1 %, control: 27.3 %). However, there were no significant differences in the number of trophoblast cells or in the inner cell mass. Experiment 2 confirmed that this increase in blastocyst development was not due to the solvent, PBS(−). In Expt. 3, addition of 10 % fetal calf serum (FCS) or ADSC lysate increased the total blastocyst rate compared to the control (control, 26.2 %; 10 % FCS, 43.4 %; 1 % ADSC lysate, 34.2 %; 10 % ADSC lysate, 48.1 %). After freezing and thawing, the survival and hatching rates of embryos with FCS were significantly lower than those of the control as well as those with added ADSC lysate. In Expt. 4, the addition of ADSC lysate or FCS had no significant effect on gene expression in blastocysts compared to control. However, the addition of FCS significantly increased the gray intensity, indicating higher lipid content compared to the control, with a significant increase in the number of dead cells in the blastocyst. These results indicate that the addition of ADSC lysate to the IVC medium accelerates bovine blastocyst development and that its 10 % addition, corresponding to 1×10^5 cells/mL, is as effective as 10 % FCS without a decrease in cryotolerance due to the increased lipid content.

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Abbreviations:

ADSCs	adipose-derived stem cells; BO medium, a medium by Brackett and Oliphant (1975)
FCS	fetal calf serummSOFaa medium, modified synthetic oviductal fluid containing amino acids reported by Takahashi et al. (1992)
PBS(−)	phosphate-buffered saline without Ca^{2+} and Mg^{2+}
IVC	*in vitro* culture

1. Introduction

In vitro production (IVP) of bovine embryos is an important technique for embryo transfer (ET) in the bovine industry and has been modified through co-culture with oviductal cells, serum supplementation and, subsequently, culture in serum-free media [1–3]. Various additives have been studied to obtain greater numbers of blastocysts such as antioxidants and growth factors [4,5]. Co-culture systems with somatic cells, such as oviductal epithelial and granulosa cells, have been studied, but they are not used widely due to their complexity. Generally, serum is often added to *in vitro* culture (IVC) media to supply bioactive substances, as serum-free media tend to result in lower blastocyst development rates [6]. On the other hand, following the discovery of mesenchymal stromal cells in bone marrow-derived [7] and adipose-derived stem cells [8], several studies have reported improved blastocyst development rates when co-cultured with bone marrow-derived [9] or adipose-derived [10] stem cells. Stem cells have a crucial role in regenerating damaged tissues, and recent research has shown that they actively secrete factors that stimulate cell division and promote regeneration in neighboring cells [11,12]. Some studies even mainly attribute the effects of stem cell transplantation to secreted factors rather than cell engraftment and proliferation [13,14]. In this context, there are a few reports on blastocyst production methods using conditioned medium [15–18]. Furthermore, cell lysates are generally used to analyze intracellular components, with cell fragmentation methods including mechanical disruption such as freeze-thaw, chemical methods, and enzymes, as outlined in a review by Ref. [19]. Following cell fragmentation, lysates are employed to investigate the expression, modification, and interaction of biomolecules, including proteins and nucleic acids [19,20]. Nevertheless, very few studies have applied stem cell lysates to culture systems or clinical treatments [20]. It could be hypothesized that stem cell lysates might have similar effects to the conditioned medium. Nonetheless, no studies have added stem cell lysates to bovine IVP thus far. Hence, this study aimed to investigate the effects of adding ADSC lysate to the culture of *in vitro* fertilized oocytes on their *in vitro* development. Here we tested the effect of ADSC lysate on *in vitro* fertilized bovine embryos. ADSC lysate accelerated development of embryos in a dose-dependent manner and improved cryotolerance of the *in vitro* fertilized embryos through reduction of lipid content.

2. Materials and methods

2.1. Preparation of bovine ADSCs and its lysate

ADSCs were isolated from the subcutaneous fat tissue around the base of the ear in an adult cow at a nearby slaughterhouse, following a slightly modified method from previous research [21]. Briefly, approximately 10 g of subcutaneous fat tissue was harvested and thoroughly washed with Medium 199 with Hanks' balanced salts (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), which contained 100 U/mL penicillin G sodium salt, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, and 0.001 % benzalkonium chloride. The tissue was minced and then digested with with 0.2 % collagenase Type I and 0.1 % dispase II in Dulbecco's Modified Eagle Medium (DMEM; Gibco™) for 120 min at 37 °C. PBS(−) supplemented with 10 % fetal calf serum (FCS) was added to the digested tissue, which was then centrifuged at 418×*g* for 5 min. The pellet was resuspended in PBS(−) and centrifuged again. After resuspending the pellet in MF-start™ medium (TOYOBO, Osaka, Japan), the cells were cultured at 38.5 °C in 5 % CO_2 in air with 100 % humidity. Upon reaching confluency, the cells were detached using 0.25 % trypsin and 0.2 % EDTA (Sigma-Aldrich, St. Louis, MO, USA), washed with PBS(−) supplemented with 10 % FCS by centrifugation. The pellet was re-suspended and seeded in MesenPro RS™ medium (Gibco™, Thermo Fisher Scientific). For later passages, cells were similarly detached, washed, and cultured in MesenPRO RS™ medium. In all experiments, ADSCs ranging from passages 3 to 8 were used.

For the preparation of ADSC lysate, confluent cells were detached using trypsin-EDTA solution as described above, rinsed with PBS(−) containing 10 % FCS, and centrifuged. The pelleted cells were resuspended in PBS(−) and the concentration was adjusted to 1×10^6 cells/mL. To disrupt the cell membrane, cells were frozen at −20 °C without a cryoprotective agent and thawed at room temperature. The suspension of disrupted cells was then centrifuged, and the supernatant was filtered through a 0.22 μm cellulose acetate membrane filter (Merck Millipore, Burlington, MA, USA). The filtrate, serving as ADSC lysate, was added to the IVC medium at concentrations of 1 and 10 %. The protein concentration in the ADSC lysate was assessed using the TaKaRa Bradford Protein Assay Kit (Takara Bio Inc., Shiga, Japan).

2.2. Preparation of bovine fibroblasts and their lysates

Auricle tissue was collected from one newborn Holstein calf that had died due to dystocia. The collected tissue was gently washed with PBS(−) containing 100 U/mL penicillin G sodium salt and 100 μg/mL streptomycin sulfate, then cut into pieces of about 1 cm^2. The auricular tissue pieces were placed onto collagen-coated dishes filled with DMEM containing 20 % FCS, 100 U/mL penicillin G sodium salt, and 100 μg/mL streptomycin sulfate and were cultured at 38.5 °C in 5 % CO_2 in air with 100 % humidity. The medium was changed daily, and the tissue was removed after 72 h. The medium was then replaced every other day until the cells that had migrated to the bottom surface became confluent as described above. For passage, upon reaching confluence, the cells were detached using trypsin-EDTA solution for 5 min. The dish was rinsed with PBS(−) containing 10 % FCS, which was combined with the trypsin-EDTA solution containing detached cells. After the collected cell suspension was centrifuged, the pelleted cells were resuspended in DMEM supplemented with 10 % FCS, 100 U/mL penicillin G sodium salt and 100 μg/mL streptomycin sulfate and cultured. The lysate was prepared using the same method as described above for ADSCs.

2.3. Immunophenotyping

ADSCs were identified following the recommendations of the International Society for Cellular Therapy (ISCT), outlining the minimal criteria for defining multipotent mesenchymal stromal cells [22]. Although markers for ADSCs in cattle have not been established, these markers have been used as criteria in various studies [10,23]. ADSCs were seeded in the wells of an 8-well chamber slide (Nunc LabTek II CC2 Chamber, Thermo Fisher Scientific) at a concentration of 1×10^4 cells/mL and cultured overnight. Cells were fixed with 4 % paraformaldehyde for 10 min. After washing with PBS(−) containing 1 % bovine serum albumin (BSA) three times, fixed cells were blocked by incubation in PBS(−) containing 5 % BSA at room temperature for 60 min. The primary antibodies were diluted in PBS(−) containing 5 % BSA at the concentrations specified below and then added to the slides and incubated overnight at 4 °C. The primary antibodies used were CD73 (Rabbit, 12231-1-AP, 1:500, Proteintech), CD105 (Rabbit, 10862-1-AP, 1:500, Proteintech, Rosemont, IL, USA), CD34 (Rabbit, 14486-1-AP, 1:800, Proteintech), CD45 (Rabbit, 20103-1-AP, 1:500, Proteintech),

and CD79a (Mouse, MA5-13212, 1:500, Invitrogen™, Thermo Fisher Scientific). The cells were then washed with PBS(−) and incubated for 60 min at room temperature with secondary antibodies diluted with PBS (−) containing 1 % BSA. The secondary antibodies used were CoraLite488-conjugated anti-rabbit IgG (Goat, SA00013-2; 1:1000, Proteintech) and FITC-conjugated anti-mouse IgG (Goat, SA00003-1; 1:200, Proteintech). After washing with PBS(−), cell nuclei were counter-stained with 10 μg/mL Hoechst 33342 (Dojindo, Kumamoto, Japan) in PBS(−) for 15 min at room temperature. Stained cells were mounted in a solution of 0.22 M 1,4-Diazabicyclo [2.2.2] octane (DABCO) in glycerol/PBS(−) (9:1) and observed under an epifluorescence microscope (BX51, U-LH100HG with WU and WIB filters, Olympus, Tokyo, Japan). Two-hundred cells were counted, and the percentage of positive cells was calculated.

2.4. IVC

Ovaries were collected from Holstein heifers or cows and their crossbreds with Japanese Black bulls at a nearby abattoir. Cumulus–oocyte complexes (COCs) were obtained from the follicles and matured in culture for 21 h Medium 199 with Earle's salts (M199; Gibco™, Thermo Fisher Scientific) supplemented with 5 % FCS, 100 U/mL penicillin G sodium salt, and 100 μg/mL streptomycin sulfate at 38.5 °C in 5 % CO_2 in air with 100 % humidity. Frozen semen from one Holstein or one Japanese Black bull was thawed at 38 °C and used for *in vitro* fertilization (IVF). Semen from the same ejaculate was used within each experiment. The thawed semen was washed by mixing it with medium (BO medium; Brackett and Oliphant, 1975 [24]) containing 5 mM caffeine monohydrate, 1.3 U/ml heparin sodium salt, and 5 mg/ml BSA (IVF100, Research Institute for the Functional Peptides Co., Yamagata, Japan) followed by two rounds of centrifugation. Spermatozoa at a final concentration of 5×10^6 spermatozoa/mL in IVF100 were used for IVF. Approximately 50 matured COCs were incubated in the sperm-containing medium for 6 h. Spermatozoa and cumulus cells were then removed by gentle pipetting, and 10–15 presumptive zygotes were transferred into modified synthetic oviductal fluid containing amino acids (mSOFaa medium) as described by Takahashi et al. [25]. The mSOFaa medium was consistently supplemented with 3 mg/mL BSA, except in cases where FCS was added. The medium also contained 100 U/mL penicillin G sodium salt and 100 μg/mL streptomycin sulfate. The zygotes were cultured for 8 days, without any medium change, at 38.5 °C in an environment with 5 % CO_2 and 90 % N_2 with 100 % humidity. Presumptive zygotes that underwent division into two or more cells on Day 2 after IVF (Day = 0) were considered cleaved. The count of embryos recognized as blastocysts was recorded on Days 6, 7, and 8.

2.5. Blastocyst cell counting

The double-staining method for simultaneously staining the trophectoderm (TE) and inner cell mass (ICM) was performed based on the protocol described by Thouas et al. [26]. Briefly, blastocysts on Day 7 after IVF were first incubated in 1 % Triton X-100 and 100 μg/mL propidium iodide diluted in M199 for up to 30 s, then immediately transferred into 25 μg/mL Hoechst 33342 in absolute ethanol and stored at 4 °C. After more than 3 h, the blastocysts were washed in glycerol, mounted on glass slides, gently flattened with a cover glass, and observed under a fluorescence microscope with a WU filter (Olympus). Photos were taken to count TE and ICM cells separately; the cell nuclei of TE were stained red and those of ICM were stained blue.

The cell membrane integrity of blastocysts was investigated based on the protocol described by Saha and Suzuki [27]. Briefly, blastocysts on Day 7 were first incubated in M199 containing 10 μg/mL propidium iodide and 10 μg/mL Hoechst 33342 for 30 min. The blastocysts were then washed in glycerol, mounted on glass slides, gently flattened with a cover glass, and observed under a fluorescence microscope with a WU filter (Olympus). Photos were taken to count the Hoechst 33342-positive and PI-positive cells (as dead cells) separately; the nuclei of the former were stained blue and those of the latter, red. Cells were counted using the Kachikachi counter (freeware, GT, Tokyo, Japan). Embryos that were lost or had poor staining quality were excluded from the count.

2.6. Cryotolerance test

The cryotolerance of blastocysts obtained from Experiment 4 (as described below) on Days 6–8 was evaluated. The embryos were transferred into cryoprotective medium composed of 0.9 M ethylene glycol, 0.8 M propylene glycol, 0.1 M sucrose, and 20 % FCS in M199. The embryos were then loaded into straws within 15 min of being placed in the cryoprotective medium. The straws were placed in a programmed freezer pre-cooled to the seeding ice temperature (−7 °C). Following a 10-min holding time after ice seeding, the straws were cooled at a rate of −0.3 °C/min until reaching −30 °C and then maintained at this temperature for 5 min before being transferred into liquid nitrogen for storage.

To assess the viability of thawed embryos, the straws were removed from liquid nitrogen, exposed to air for 6–10 s, and then placed in warm water at 30 °C. The embryos were transferred into M199 supplemented with 20 % FCS, 100 U/mL penicillin G sodium salt, and 100 μg/mL streptomycin sulfate and cultured at 38.5 °C in 5 % CO_2 in air with 100 % humidity. Morphological assessments were performed to evaluate viability at 0, 3, 24, 48, and 72 h after thawing. Embryos were deemed viable if cell aggregation was maintained or if the blastocoel was re-expanded or maintained at each evaluation time. The hatched embryos were then counted 72 h after thawing.

2.7. Real-time RT- PCR

The blastocysts on Day 7 were rinsed three times with PBS(−), and finally transferred into a minimal amount of saline. Total RNA was then isolated from a pool of five embryos using the RNeasy Micro Kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's instructions. Total RNA was used to synthesize cDNA through the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The total RNA, along with the buffer mix and enzyme mix, was incubated for 60 min at 37 °C and 5 min at 95 °C, then cooled to 4 °C. The cDNA samples were frozen and kept at −80 °C until real-time RT-PCR.

Real-time RT-PCR was performed to assess the expression levels of *POU5F1* (POU class 5 homebox 1), *BAX* (BCL2 associated X, apoptosis regulator), *IGF1R* (insulin-like growth factor 1 receptor), *IGF2R* (insulin-like growth factor 2 receptor), and *SOD2* (superoxide dismutase 2) relative to the housekeeping gene *PPIA* (Peptidylprolyl isomerase A). Quantitative analyses of targeted transcripts were performed using TaKaRa TB Green® Premix Ex Taq™ II on a TaKaRa Thermal Cycler Dice Realtime System III (Takara Bio Inc). Real-time RT-PCR reactions were carried out in a total volume of 25 μl following the manufacturer's instructions. The cycling parameters were 2 min at 95 °C for denaturation, 40 cycles of 5 s at 95 °C, 30 s at 60 °C for amplification and quantification. Specific primers sequences are listed in Table 1. For the internal control genes, *PPIA* and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) were initially used, with *PPIA* ultimately chosen for its stability. Quantitative evaluation of target gene expression relative to the internal standard gene expression level was calculated using the ΔΔct method.

2.8. Evaluation of lipid content of blastocysts

Lipid content in blastocysts was evaluated using Sudan Black, as described by Sudano et al. [28] and Dias et al. [29]. Briefly, blastocysts were washed in PBS(−) and refrigerated PBS(−) containing 3.7 w/v % formaldehyde at 4 °C until evaluation. The blastocysts were washed twice in Milli-Q water containing 0.05 % PVA (PVA-H_2O) and then

Table 1
Primers used for real-time RT-PCR quantitative analysis.

Gene	Sequences		Accession number
PPIA	forward	TGGTGACTTCACACGCCATAA	XM001252497
	reverse	GATGCCAGGACCTGTATGCTTC	
POU5F1	forward	CTGCTGCAGAAGTGGGTGGA	NM_174580.3
	reverse	CTCACTCGGTTCTCGATACTCGTC	
BAX	forward	AGCGCATCGGAGATGAATTG	NM_173894.1
	reverse	CCAGTTGAAGTTGCCGTCAG	
IGF2R	forward	GCCAGCTGGTGAAGTCAAACAA	NM_174352.2
	reverse	AGACACACATGTGCGCACCTA	
IGF1R	forward	CGGTCTCTGAGGCCAGAAATG	NM_001244612.1
	reverse	CAGCGAGGTCTCTGTGGACAA	
SOD2	forward	AGCCCTAACGGTGGTGGAGA	NM_201527.2
	reverse	GGAGCCTTGGACACCAACAGA	
YWHAZ	forward	CGAGCAGGCTGAGCGATATG	NM_174814.2
	reverse	AAGATGACCTACGGGCTCCTACAAC	

POU5F1 (POU class 5 homebox 1), *BAX* (BCL2 associated X, apoptosis regulator), IGF1R (insulin-like growth factor 1 receptor), *IGF2R* (insulin-like growth factor 2 receptor), *SOD2* (superoxide dismutase 2), and *PPIA* (TRIM5/cyclophilin A fusion protein-like, transcript variant 1), *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta).

transferred to a well loaded with PVA-H_2O containing 50 % ethanol for 2 min. They were then stained with 1 % Sudan Black in 70 % ethanol for 2 min, followed by three 5-min washes in PVA-H_2O with 50 % ethanol. The embryos were then washed 2–3 times in PVA-H_2O and mounted on a slide, covered with a cover slip, and examined under an inverted microscope (Olympus). Each embryo was photographed, and the photographs were evaluated for color intensity using Image J 1.41 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). For the analysis, the colored images were converted to greyscale and then inverted. The gray intensity of the area within the embryo cropped around the zona pellucida was quantified as the mean gray intensity in arbitrary units (indicative of lipid content).

2.9. Experimental design

2.9.1. Experiment 1. evaluation of the effects of ADSC and fibroblast lysates on embryo development

We evaluated the effects of lysates derived from two different cell types, ADSCs and fibroblasts (used as a control to ADSCs) on embryo development. The mSOFaa medium, containing 3 mg/ml BSA without the addition of any lysate, was used as a control, to which lysates were added. Cleavage was recorded on Day 2, and blastocyst development was recorded on Days 6, 7, and 8 of culture. The percentages of cleaved zygotes on Day 2 and of blastocysts on Days 6, 7, and 8 in relation to the initial number of presumptive zygotes undergoing IVC were calculated. The blastocysts produced were taken to count the TE and ICM cells on Day 7.

2.9.2. Experiment 2. comparison of the effects of ADSC lysate and its solvent on embryo development

A comparison was conducted between the group using only 10 % PBS (−) as the solvent control and the group with 10 % ADSC lysate to evaluate embryo development. The numbers of putative zygotes cleaved on Day 2 and of blastocysts formed on Days 6, 7, and 8 were recorded. The percentages of putative zygotes cleaved on Day 2 and of blastocysts on Days 6–8 in comparison to the number of putative zygotes undergoing IVC were obtained.

2.9.3. Experiment 3. dose-dependent effects of ADSC lysate on development and cryotolerance of embryos and comparison with those of FCS

To determine the optimal concentration of ADSC lysate, different concentrations (0 %, 1 %, and 10 %) of ADSC lysate (equivalent to 1 × 10^6 cells/ml) added to mSOFaa including 0.3 % BSA were compared. FCS was integrated at a 10 % concentration in the mSOFaa for comparison with the ADSC lysate. Cleavage was recorded on Day 2, and blastocyst development was recorded on Days 6, 7, and 8 of culture. The percentages of embryos in the blastocyst and expanded blastocyst stages on Days 6–8 in relation to the initial number of presumptive zygotes undergoing IVC were obtained. Embryos at the blastocyst to expanded blastocyst stages obtained on Days 6–8 were used for the cryotolerance test. The count of blastocysts, encompassing those removed for cryotolerance testing during the 8 days of IVC, was recorded.

2.9.4. Experiment 4. analyses of blastocysts by membrane integrity of the cell, lipid content, and gene expression

To examine embryo quality in more detail, blastocysts were produced as described above by IVC in mSOFaa medium supplemented with 0.3 % BSA (as control), 10 % FCS, or 10 % ADSC lysate using 675 immature oocytes. The blastocysts were subjected to evaluation of cell membrane integrity (74 embryos), determination of lipid content (62 embryos) and gene expression analysis (45 embryos) following the procedure outlined earlier.

2.10. Statistical analyses

Data are presented as means ± SEM. After arcsine transformation (arcsin $\sqrt{}$%/100), the Shapiro–Wilk test was performed to confirm normal distribution. ANOVA and Tukey's test were then performed to compare the mean percentages of cleavage on Day 2 and of blastocysts on Days 6–8. The cell count of blastocysts underwent the Shapiro–Wilk test to assess its adherence to a normal distribution. Because the cell count did not follow a normal distribution, the Kruskal–Wallis test was performed. To compare the results of the cryotolerance test, Fisher's exact test with Holm correction was conducted. To analyze the gray intensity of the embryo image and mRNA expression, the Shapiro–Wilk test was performed to confirm normal distribution, followed by ANOVA and Tukey's test for further analysis. R version 4.2.3 software (R Foundation, Vienna, Austria) was used for all the statistical analyses.

3. Results

In order to confirm that the isolated cells are ADSCs after culture, morphological features and expression of mesenchymal markers (CD73 and CD105) and hematopoietic markers (CD34, CD45 and CD79a) were check by immunostaining.

Cells isolated from subcutaneous fat tissue showed a fibroblast-like morphology characterized by a singular nucleus. The cells tested positive for the mesenchymal markers CD73 (92.5 %) and CD105 (94.9 %) while being negative for the hematopoietic markers CD34 (0 %), CD45 (0 %), and CD79a (0 %) (Fig. 1).

The protein concentrations in the lysates were determined to ascertain the quality of the lysate and were 246.9 μg/ml for ADSC lysate and 268.9 μg/ml for the fibroblast lysate, showing that the concentration was reasonably high.

Experiment 1 examined the effect of lysate of ADSC and fibroblast on bovine embryo development *in vitro*. The percentages of cleaved zygotes on Day 2 were not significantly different among the different cell types from which lysate was derived. The addition of ADSC or fibroblast lysate resulted in a significantly higher percentage of blastocysts on Day 7 compared to the respective control conditions, suggesting that both lysates have promoting effect on embryo development. Although not statistically significant, the rate of blastocyst tended to be greater for the ADSC lysate than for fibroblast lysate. The percentages of blastocysts on Days 6 and 8 were also significantly higher when ADSC lysate was added compared to the control setup (Fig. 2), suggesting that the promoting effect of the ADSC lysate manifests itself earlier than fibroblast lysate.

In order to reveal the quality of embryos produced by using the lysate, cell numbers and viability of the TE and ICM was examined. Double staining for the numbers of TE and ICM cells, as well as the overall cell count, exhibited no significant variances among the control

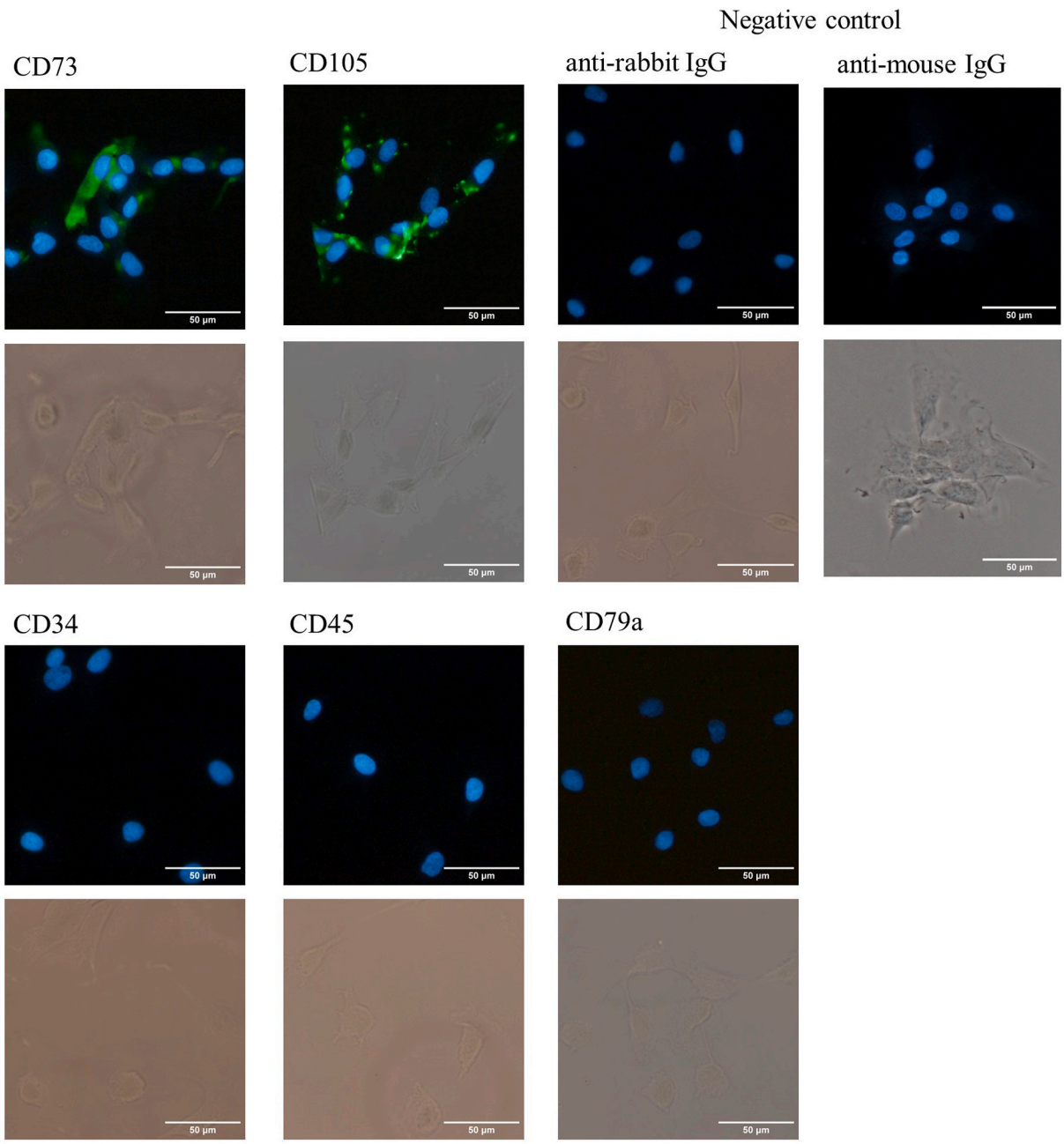

Fig. 1. Localization of surface markers in ADSCs (adipose-derived stem cells, Passage 4) revealed by immunophenotyping. Positive surface markers are shown in green and nuclei in blue. Mesenchymal markers CD73 and CD105 are positive, whereas hematopoietic markers CD34, CD45, and CD79a are negative. Scale bar indicates 50 μm. Negative controls: Cells were incubated solely with the 2nd antibody of CoraLite488-conjugated anti-rabbit or FITC-conjugated anti-mouse IgG without incubation with the 1st antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(0.3 w/v % BSA), fibroblasts lysate (10 %), and ADSC lysate (10 %) groups, as shown in Table 2. The results suggest that the quality of the embryo produced is similar among the lysates of ADSC and fibroblast and BSA and that the lysate of ADSC and fibroblast does not increase the cell number constituting the produced blastocyst compared to the control (BSA).

Because the effect of the lysate was not discriminated from that of the solvent PBS(−) in Experiment 1, the Experiment 2 compared embryo development between 10 % PBS(−) alone and the lysate of ADSC. The rates of blastocyst from Days 6–8 were significantly higher with 10 % ADSC lysate compared to the control lacking ADSC lysate (10 % PBS(−)) (Table 3), confirming that the effect of the lysate arises from ADSC-derived factors.

In Experiment 3, dose-dependence of the ADSC lysate was investigated with comparison to a conventional additive of FCS. The addition of 10 % FCS or 10 % ADSC lysate into the medium was found to significantly ($P < 0.01$) increase blastocyst development rates compared to the control across Days 6, 7, and 8 (Fig. 3). The addition of 1 % ADSC lysate also resulted in a higher percentage of blastocysts than the control on Day 7; this percentage was significantly lower than that observed with the addition of 10 % ADSC lysate (Fig. 3). The results show that the promoting effect of ADSC lysate on embryo development is dose-dependent with 10 % ADSC lysate paralleling that of 10 % FCS.

In order to characterize the embryo produced by using ADSC lysate, embryos obtained in Experiment 3 were also examined for their cryotolerance. The inclusion of ADSC lysate notably enhanced the percentage of post-thaw survival at 24, 48, and 72 h, matching the outcome with BSA alone and surpassing that of FCS (Table 4), irrespective of its

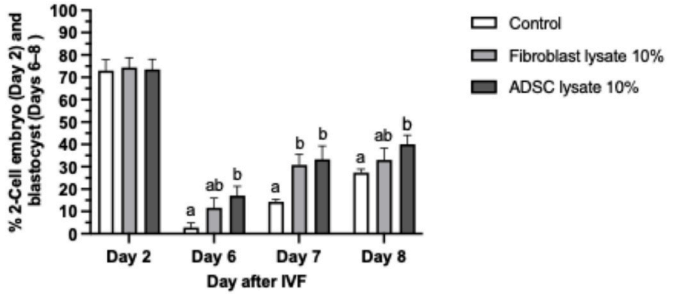

Fig. 2. Proportion (%, mean ± SEM) of cleaved zygotes on Day 2 and of blastocysts on Days 6, 7 and 8 in relation to the initial number of presumptive zygotes undergoing IVC in Experiment 1. BSA (3 mg/ml) was added to mSOFaa (control). ADSC or fibroblast lysate was added at a concentration of 10 % to the BSA-supplemented mSOFaa during culture. The experiment was repeated five times, using 152 oocytes in each group, for a total of 456 oocytes.
a, b: Values with different superscripts differ significantly within the column ($P < 0.05$ by Tukey's test following ANOVA).

concentration (1 % or 10 %). The ratio of blastocysts that hatched after thawing was significantly higher for both the 1 % and 10 % ADSC lysate compared to 10 % FCS, which in turn was significantly lower than BSA (Table 4).

To clarify the reason for the improved cryotolerance of embryos obtained by ADSC lysate, viability of the cell constituting the blastocyst, lipid content and gene expressions of the blastocysts were examined in Experiment 4. The tally of PI-positive cells (nonviable cells) was significantly higher when FCS was added compared to the cases involving BSA with or without ADSC lysate whereas the count of H33342-positive cells (viable cells) displayed no significant variations among the three treatments (Table 5), suggesting that ADSC lysate improved cell viability rather cell proliferation compared to FCS.

Analysis of the lipid content of blastocysts revealed that the mean gray intensity was lowest with the addition of BSA alone, followed by the addition of ADSC lysate, and was highest with FCS (Table 5), suggesting that lipid content in blastocysts with ADSC lysate is lower than that with FCS but higher than that with BSA.

No variations in mRNA expression were detected in blastocysts for *BAX*, *IGF1R*, *IGF2R*, *POU5F1*, and *SOD2*. However, *IGF2R* tended to be higher with FCS addition compared to BSA ($P = 0.053$) (Fig. 4). The results suggest that gene expression was not remarkably affected by either ADSC lysate or FCS.

4. Discussion

Immunophenotyping results confirmed that ADSCs were positive for the mesenchymal cell markers suggested by the International Society for Cell and Gene Therapy but negative for all hematopoietic markers [22]. Miranda et al. [10] reported that bovine ADSCs were immunocytochemically positive for CD90, 75, and 105 and negative for CD34, 45, and 79. Our results were in agreement with that report, validating the identity of the ADSCs used in this research.

Our study demonstrated for the first time that the supplementation of ADSC lysate to IVC medium increases the blastocyst development rate of bovine embryos without affecting blastocyst quality in terms of TE and ICM cell counts. The addition of either ADSC lysate or FCS to IVC medium was found to hasten progression to the blastocyst stage (Table 3, Figs. 2 and 3), with Experiment 2 affirming that this increase in blastocyst development was not due to the solvent, PBS(−). The growth-promoting effect of 10 % ADSC lysate equivalent to 10^5 ADSCs/ml was comparable to that of 10 % FCS (Fig. 3). The addition of FCS or high concentrations (16 mg/ml) of BSA to IVC medium has been reported to hasten the blastocyst stage [3]. Culturing on a monolayer of bovine ADSCs as feeder cells hastened blastocyst development [10], and a conditioned medium from human ADSCs promoted the development of porcine embryos *in vitro* [18,30]. This study is the first to incorporate ADSC lysate for the IVC of bovine embryos. The lysate's advantage is that it can be stored frozen until use and is readily employed without the

Table 3
Proportions (mean ± SEM, %) of the accumulated numbers of cleaved zygotes on Day 2 and of blastocysts on Days 6–8 of IVC in the presence or absence of 10 % ADSC lysate in Experiment 2.

Group		No. of replicates	No. of putative zygotes cultured	% of zygotes cleaved on Day 2	% of blastocysts*		
PBS (−)	ADSC lysate				Day 6	Day 7	Day 8
+	–	5	291	85.8 ± 2.8	0.5 ± 0.3[a]	10.5 ± 2.3[a]	29.9 ± 4.6[a]
–	+	5	286	90.1 ± 2.3	8.3 ± 1.7[b]	22.0 ± 4.3[b]	43.0 ± 4.0[b]

a, b: Values with different superscripts differ significantly within the column by paired *t*-test.
*Proportion to the number of putative zygotes cultured.

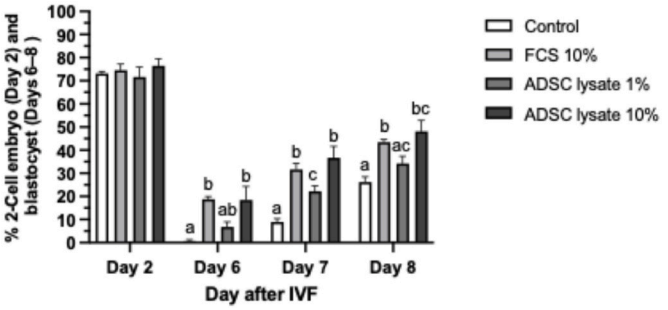

Fig. 3. Proportion (%, mean ± SEM) of putative zygotes cleaved on Day 2 and of embryos at the blastocyst and expanded blastocyst stages on Days 6–8 in relation to the initial number of presumptive zygotes undergoing IVC in Experiment 3. Proportion (%, mean ± SEM) in Experiment 3. BSA was added to mSOFaa as a control. ADSC lysate was added at a concentration of 1 % or 10 % to the BSA-supplemented mSOFaa (control) during IVC. FCS was added at a concentration of 10 % to mSOFaa without BSA. The experiment was repeated five times, using 192 oocytes in each group, for a total of 768 oocytes.
a–c: Values with different superscripts differ significantly within the column ($P < 0.05$ by Tukey's test following ANOVA).

Table 2
Counts of blastocyst cells formed after IVC in the presence or absence of ADSC lysate or fibroblast lysate in Experiment 1.

Group			Blastocyst staining				
			No. of cells counted				
BSA	ADSC lysate	Fibroblast lysate	No. of blastocysts examined	No. of blastocysts with poor staining	TE	ICM	Total
+	–	–	17	3	62.5 ± 26.1	38.3 ± 10.9	100.8 ± 30.1
+	+	–	41	12	77.8 ± 33.7	40.0 ± 12.7	117.9 ± 38.0
+	–	+	38	10	68.4 ± 32.5	41.5 ± 12.7	109.9 ± 35.7

No significant differences in TE, ICM, or total cell counts were found among the groups within the column by the Kruskal–Wallis test.

Table 4
Results of cryotolerance test results in Experiment 3.

Group			No. of blastocysts examined	Survival number (%) after culture for:					No. (%) of blastocysts hatched
BSA (0.3 %)	FCS (10 %)	ADSC lysate (%)		0 h	3 h	24 h	48 h	72 h	
+	–	0	19	19 (100)	18 (94.7)	17 (89.5)[a]	17 (89.5)[a]	17 (89.5)[a]	14 (73.7)[a]
–	+	0	25	23 (92.0)	21 (84.0)	13 (52.0)[b]	9 (36.0)[b]	8 (32.0)[b]	6 (24.0)[b]
+	–	1	21	21 (100)	21 (100)	18 (85.7)[a]	17 (80.9)[a]	17 (80.9)[a]	15 (71.4)[a]
+	–	10	35	33 (94.2)	33 (94.2)	31 (88.6)[a]	27 (77.1)[a]	27 (77.1)[a]	22 (62.9)[a]

a, b: Values with different superscripts differ significantly within the column by Fisher's exact test with Holm correction ($P < 0.05$).

Table 5
Cell integrity (cell staining) and lipid content (lipid staining) of blastocysts formed after IVC in the presence or absence of fibroblast or ADSC lysate in Experiment 4.

Group			Cell staining (Cell number ± SEM of positive cells)			Lipid staining	
BSA (0.3 %)	FCS (10 %)	ADSC lysate (10 %)	No. of blastocysts examined	Hoechst 33342	PI	No. of blastocysts examined	Mean gray intensity ± SEM (arbitrary units)
+	-	-	16	108.2 ± 24.7	2.4 ± 2.8[a]	18	104.4 ± 11.1[a]
-	+	-	24	113.5 ± 46.5	5.0 ± 5.2[b]	24	147.6 ± 16.9 [b]
+	-	+	34	118.3 ± 33.0	1.2 ± 1.7[a]	20	126.1 ± 15.9[c]

a, b (Cell staining): Values with different superscripts differ significantly within the column ($P < 0.05$ by Kruskal–Wallis test).
a–c (Lipid staining): Values with different superscripts differ significantly within the column ($P < 0.05$ by Tukey's test following ANOVA).

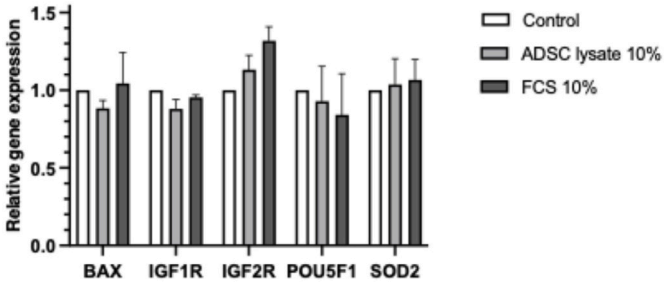

Fig. 4. Relative gene expression in the gene expression analysis of *in vitro* produced bovine embryos in the presence or absence of 10 % FCS or 10 % ADSC lysate. Relative gene expression of *BAX* (A), *IGF1R* (B), *IGF2R* (C), *POU5F1* (D), and *SOD2* (E) was analyzed in blastocysts cultured in the SOFaa medium containing 0.3 % BSA (as a control), 10 % FCS, and 10 % ADSC lysate. No significant difference was observed among the three groups ($P > 0.05$ by paired *t*-test). A slight trend of higher expression of the *IGF2R* gene was noted in the presence of FCS compared with BSA ($P = 0.053$ by Tukey's test following ANOVA).

necessity for prior culture of growing cells or harvesting of culture medium.

It is known that the addition of FCS to *in vitro* culture media enhances the blastocyst yield [6]. Nevertheless, it is widely recognized that relatively high concentrations of glucose in FCS inhibit oocyte cleavage while promoting blastocyst development [25]. Moreover, FCS has been linked to an increased risk of abnormal offspring [31,32]. It has also been reported that the addition of FCS increases apoptosis in porcine blastocysts [33], aligning with the increase in nonviable cells observed in this study (Table 5). *In vitro* fertilized bovine blastocysts cultured in the presence of FCS contained more lipid [34], suggesting a connection between increased lipid levels in blastocysts and reduced cryotolerance [29,35]. The present results showed that the addition of FCS increased the lipid content in blastocysts, dramatically reducing their cryotolerance, as evidenced by embryo survival and hatching after thawing (Table 4), compared to BSA alone. Conversely, the addition of 1 % or 10 % ADSC lysate reduced lipid content substantially, significantly below that of FCS in comparison to BSA alone (Table 5), while showing superior cryotolerance over FCS (Table 4). Thus, it is suggested that the addition of ADSC lysate increases the cryotolerance of *in vitro* fertilized bovine embryos by reducing lipid content with a blastocyst formation rate equal to that of FCS (10 %).

The gene expression analyses in this study revealed no significant difference in the gene expression levels of *BAX*, *SOD2*, *IGF1R*, *IGFR2*, and *POU5F1* when comparing the addition of BSA alone, FCS, and BSA with ADSC lysate. This suggests that the embryos developed in the presence of ADSC lysate exhibit gene expression profiles akin to those developed with BSA or FCS. Differences in expression levels of other genes, however, were not clear in this study.

SOD2 is a marker for oxidative stress to which embryos have been exposed [36,37]. The absence of a difference in the expression levels of *SOD2* showed that oxidative stress was present at similar levels across all treatments. *Bax* is an inducer of apoptosis in embryos [3]. Expression of *Bax* genes did not differ among the treatments in this study, whereas the number of dead cells was significantly higher for FCS compared to ADSC lysate. Rizos et al. [3] reported that the presence of serum significantly increased the gene expression of *SOD2* compared to its absence, while the expression of *BAX* decreased in the absence of serum. Our results may have diverged due to the fact that embryos were cultured under low oxygen conditions in this study, potentially resulting in lower oxidative stress compared to the experiments conducted by Rizos et al. [3], where *in vitro* culture was not carried out under low oxygen conditions. The absence of variance in the expression levels of *IGF1R* genes between the embryos cultured with FCS and BSA in this study aligns with the finding by Lazzari et al. [38]. The addition of ADSC lysate did not result in a difference in the expression of *IGF1R* compared to that with FCS. In contrast, cryotolerance differed significantly different between FCS and ADSC lysates in this study. Therefore, it did not seem that cryotolerance was associated with the expression levels of *IGF1R*.

Although there was no significant difference in gene expression levels of *IGF2R*, a slight trend toward higher expression of *IGF2R* genes with the addition of FCS was noted ($P = 0.053$). In general, there is disagreement regarding the interpretation of *IGF2R* expression in embryos. Young et al. reported low *IGF2R* expression in the tissues of Large Offspring Syndrome (LOS) infants [31], whereas there are reports that embryos produced *in vitro* and *in vivo* exhibit comparable levels of *IGF2R* gene expression [38,39]. Sugimura et al. demonstrated that low *IGF2R*

expression is related to slow growth and reduced fertility in embryos, as observed in LOS [40]. The present results regarding *IGF2R* might reflect a higher developmental pace.

The expression of *POU5F1* in blastocysts is crucial for maintaining their pluripotency and for initiating the development of early embryonic structures. An elevation in *POU5F1* expression through co-culture or the addition of conditioned medium from ADSCs has been indicated [10,18,30]. However, the present study did not reveal any significant difference among the additives BSA, FCS, and ADSC lysate. This discrepancy in findings may be attributed to variations in species or to differences in culture conditions involving cell lysate.

In the initial stages of embryonic development, the preimplantation blastocyst necessitates a variety of factors for its growth and development. These factors are secreted by both the blastocyst itself and the oviductal epithelium [41,42]. Various attempts have been made to use growth factors and antioxidants to improve culture media. For example, IGF-1, IGF-2 [41], TGFα [41], TGFβ [5,41], and SOD [43,44] have been shown to be beneficial for *in vitro* embryo production. Mesenchymal stem cells, such as ADSCs, are known to secrete a wide range of growth factors, cytokines, and antioxidants [45–47]. The present study did not attempt to identify the factors present in the lysate that are responsible for the accelerated growth of embryos. Further research is needed to elucidate the mechanism underlying the action of the ADSC lysate.

Our results demonstrate, for the first time, the potential for the application of ADSC lysate as a new supplemental material that is as effective as FCS in blastocyst development. This approach addresses the drawbacks of FCS, particularly in terms of dead cell count and low cryotolerance. Moreover, the utilization of ADSC lysate offers a novel perspective in mitigating the limitations associated with FCS in terms of cryotolerance. Thus, it is expected that ADSC lysate contributes much to increasing of cattle production by ET of fresh or cryopreserved *in vitro* fertilized embryos. Knowledge obtained from this study could be applied to the assisted reproductive technology in humans.

CRediT authorship contribution statement

Noriyoshi Manabe: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Yoichiro Hoshino:** Validation, Methodology. **Takehiro Himaki:** Validation. **Kenichiro Sakaguchi:** Writing – review & editing. **Seiji Matsumoto:** Writing – review & editing, Project administration. **Tokunori Yamamoto:** Writing – review & editing, Methodology. **Tetsuma Murase:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

Tokunori Yamamoto is President of Meis Technology, Inc., which possesses a patent related to this work (Japan Patent No. 6970853). The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] M.-A. Sirard, 40 years of bovine IVF in the new genomic selection context, Reproduction 156 (2018) R1–R7, https://doi.org/10.1530/REP-18-0008.
[2] L.B. Ferré, M.E. Kjelland, A.M. Taiyeb, F. Campos-Chillon, P.J. Ross, Recent progress in bovine in vitro-derived embryo cryotolerance: impact of in vitro culture systems, advances in cryopreservation and future considerations, Reprod. Domest. Anim. 55 (2020) 659–676, https://doi.org/10.1111/rda.13667.
[3] D. Rizos, A. Gutiérrez-Adán, S. Pérez-Garnelo, J. De La Fuente, M.P. Boland, P. Lonergan, Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression, Biol. Reprod. 68 (2003) 236–243, https://doi.org/10.1095/biolreprod.102.007799.
[4] M. Takahashi, T. Nagai, S. Hamano, M. Kuwayama, N. Okamura, A. Okano, Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos, Biol. Reprod. 49 (1993) 228–232, https://doi.org/10.1095/biolreprod49.2.228.
[5] H. Abe, S. Yamashita, T. Satoh, H. Hoshi, Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media, Mol. Reprod. Dev. 61 (2002) 57–66, https://doi.org/10.1002/mrd.1131.
[6] A. Van Langendonckt, I. Donnay, N. Schuurbiers, P. Auquier, C. Carolan, A. Massip, F. Dessy, Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium, J. Reprod. Fertil. 109 (1997) 87–93, https://doi.org/10.1530/jrf.0.1090087.
[7] M. Tavassoli, W.H. Crosby, Transplantation of marrow to extramedullary sites, Science 161 (1968) 54–56, https://doi.org/10.1126/science.161.3836.54.
[8] P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H. P. Lorenz, M.H. Hedrick, Multilineage cells from human adipose tissue: implications for cell-based therapies, Tissue Eng. 7 (2001) 211–228, https://doi.org/10.1089/107632701300062859.
[9] I.J. Ascari, S.C. Martins, L.S.A. Camargo, R. Mendez-Otero, Jasmin, Development of bovine embryos in vitro in coculture with murine mesenchymal stem cells and embryonic fibroblasts, Mol. Biol. Rep. 45 (2018) 1827–1837, https://doi.org/10.1007/s11033-018-4329-y.
[10] M.S. Miranda, H.S. Nascimento, M.P.R. Costa, N.N. Costa, K.N.L. Brito, C.T. A. Lopes, S.S.D. Santos, M.S. Cordeiro, O.M. Ohashi, Increasing of blastocyst rate and gene expression in co-culture of bovine embryos with adult adipose tissue-derived mesenchymal stem cells, J. Assist. Reprod. Genet. 33 (2016) 1395–1403, https://doi.org/10.1007/s10815-016-0779-0.
[11] A.J.B.O.G. Salgado, R.L.G. Reis, N.J.C. Sousa, J.M. Gimble, Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine, Curr. Stem Cell Res. Ther. 5 (2010) 103–110, https://doi.org/10.2174/157488810791268564.
[12] H. Skalnikova, J. Motlik, S.J. Gadher, H. Kovarova, Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines, Proteomics 11 (2011) 691–708, https://doi.org/10.1002/pmic.201000402.
[13] G. Maguire, Stem cell therapy without the cells, Commun. Integr. Biol. 6 (2013) e26631, https://doi.org/10.4161/cib.26631.
[14] S.R. Baglio, D.M. Pegtel, N. Baldini, Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy, Front. Physiol. 3 (2012) 359, https://doi.org/10.3389/fphys.2012.00359.
[15] T. Fujita, H. Umeki, H. Shimura, K. Kugumiya, K. Shiga, Effect of group culture and embryo-culture conditioned medium on development of bovine embryos, J. Reprod. Dev. 52 (2006) 137–142, https://doi.org/10.1262/jrd.16084.
[16] G. Moshkdanian, S.N. Nematollahi-Mahani, F. Pouya, A. Nematollahi-Mahani, Antioxidants rescue stressed embryos at a rate comparable with co-culturing of embryos with human umbilical cord mesenchymal cells, J. Assist. Reprod. Genet. 28 (2011) 343–349, https://doi.org/10.1007/s10815-010-9529-x.
[17] R. Lopera-Vásquez, M. Hamdi, B. Fernandez-Fuertes, V. Maillo, P. Beltrán-Breña, A. Calle, A. Redruello, S. López-Martín, A. Gutierrez-Adán, M. Yañez-Mó, M.Á. Ramirez, D. Rizos, Extracellular vesicles from BOEC in in vitro embryo development and quality, PLoS One 11 (2016) e0148083, https://doi.org/10.1371/journal.pone.0148083.
[18] H.-Y. Park, E.-Y. Kim, S.-E. Lee, H.-Y. Choi, J.J. Moon, M.-J. Park, Y.-J. Son, J.-B. Lee, C.-J. Jeong, D.-S. Lee, K.-J. Riu, S.-P. Park, Effect of human adipose tissue-derived mesenchymal-stem-cell bioactive materials on porcine embryo development, Mol. Reprod. Dev. 80 (2013) 1035–1047, https://doi.org/10.1002/mrd.22270.
[19] M. Danaeifar, New horizons in developing cell lysis methods: a review, Biotechnol. Bioeng. 119 (2022) 3007–3021. https://doi.org/10.1002/bit.28198.
[20] M. Albersen, T.M. Fandel, G. Lin, G. Wang, L. Banie, C.-S. Lin, T.F. Lue, Injections of adipose tissue-derived stem cells and stem cell lysate improve recovery of erectile function in a rat model of cavernous nerve injury, J. Sex. Med. 7 (2010) 3331–3340, https://doi.org/10.1111/j.1743-6109.2010.01875.x.
[21] J. Gimble, F. Guilak, Adipose-derived adult stem cells: isolation, characterization, and differentiation potential, Cytotherapy 5 (2003) 362–369, https://doi.org/10.1080/14653240310003026.
[22] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, Cytotherapy 8 (2006) 315–317, https://doi.org/10.1080/14653240600855905.
[23] R.V. Sampaio, M.R. Chiaratti, D.C.N. Santos, F.F. Bressan, J.R. Sangalli, A.L.A. Sá, T.V.G. Silva, N.N. Costa, M.S. Cordeiro, S.S.D. Santos, C.E. Ambrosio, P.R. Adona, F.V. Meirelles, M.S. Miranda, O.M. Ohashi, Generation of bovine (Bos indicus) and

buffalo (Bubalus bubalis) adipose tissue derived stem cells: isolation, characterization, and multipotentiality, Genet. Mol. Res. 14 (2015) 53–62, https://doi.org/10.4238/2015.January.15.7.
[24] B.G. Brackett, G. Oliphant, Capacitation of rabbit spermatozoa in vitro, Biol. Reprod. 12 (1975) 260–274, https://doi.org/10.1095/biolreprod12.2.260.
[25] Y. Takahashi, N.L. First, In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins, Theriogenology 37 (1992) 963–978, https://doi.org/10.1016/0093-691x(92)90096-a.
[26] G.A. Thouas, N.A. Korfiatis, A.J. French, G.M. Jones, A.O. Trounson, Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts, Reprod. Biomed. Online 3 (2001) 25–29, https://doi.org/10.1016/s1472-6483(10)61960-8.
[27] S. Saha, T. Suzuki, Vitrification of in vitro produced bovine embryos at different ages using one- and three-step addition of cryoprotective additives, Reprod. Fertil. Dev. 9 (1997) 741–746, https://doi.org/10.1071/r97024.
[28] M.J. Sudano, V.G. Santos, A. Tata, C.R. Ferreira, D.M. Paschoal, R. Machado, J. Buratini, M.N. Eberlin, F.D.C. Landim-Alvarenga, Phosphatidylcholine and sphingomyelin profiles vary in Bos taurus indicus and Bos taurus taurus in vitro- and in vivo-produced blastocysts, Biol. Reprod. 87 (2012) 130, https://doi.org/10.1095/biolreprod.112.102897.
[29] L.R.O. Dias, L.O. Leme, J.F.W. Sprícigo, I. Pivato, M.A.N. Dode, Effect of delipidant agents during in vitro culture on the development, lipid content, gene expression and cryotolerance of bovine embryos, Reprod. Domest. Anim. 55 (2020) 11–20, https://doi.org/10.1111/rda.13579.
[30] S.-E. Lee, J.J.-M. Moon, E.-Y. Kim, S.-P. Park, Stem cell-derived bioactive materials accelerate development of porcine in vitro-fertilized embryos, Cell. Reprogram. 17 (2015) 181–190, https://doi.org/10.1089/cell.2014.0110.
[31] L.E. Young, K.D. Sinclair, I. Wilmut, Large offspring syndrome in cattle and sheep, Rev. Reprod. 3 (1998) 155–163, https://doi.org/10.1530/ror.0.0030155.
[32] P.W. Farin, J.A. Piedrahita, C.E. Farin, Errors in development of fetuses and placentas from in vitro-produced bovine embryos, Theriogenology 65 (2006) 178–191, https://doi.org/10.1016/j.theriogenology.2005.09.022.
[33] X.-S. Cui, Y.-J. Jeong, H.-Y. Lee, S.-H. Cheon, N.-H. Kim, Fetal bovine serum influences apoptosis and apoptosis-related gene expression in porcine parthenotes developing in vitro, Reproduction 127 (2004) 125–130, https://doi.org/10.1530/rep.1.00039.
[34] E.M. Ferguson, H.J. Leese, Triglyceride content of bovine oocytes and early embryos, J. Reprod. Fertil. 116 (1999) 373–378, https://doi.org/10.1530/jrf.0.1160373.
[35] M.J. Sudano, D.M. Paschoal, T. da S. Rascado, L.C.O. Magalhães, L.F. Crocomo, J. F. de Lima-Neto, F. da C. Landim-Alvarenga, Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification, Theriogenology 75 (2011) 1211–1220, https://doi.org/10.1016/j.theriogenology.2010.11.033.
[36] S. Balasubramanian, W.J. Son, B.M. Kumar, S.A. Ock, J.G. Yoo, G.S. Im, S.Y. Choe, G.J. Rho, Expression pattern of oxygen and stress-responsive gene transcripts at various developmental stages of in vitro and in vivo preimplantation bovine embryos, Theriogenology 68 (2007) 265–275, https://doi.org/10.1016/j.theriogenology.2007.05.044.
[37] A. Cebrian-Serrano, I. Salvador, E. García-Roselló, E. Pericuesta, S. Pérez-Cerezales, A. Gutierrez-Adán, P. Coy, M.A. Silvestre, Effect of the bovine oviductal fluid on in vitro fertilization, development and gene expression of in vitro-produced bovine blastocysts, Reprod. Domest. Anim. 48 (2013) 331–338, https://doi.org/10.1111/j.1439-0531.2012.02157.x.
[38] G. Lazzari, C. Wrenzycki, D. Herrmann, R. Duchi, T. Kruip, H. Niemann, C. Galli, Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome, Biol. Reprod. 67 (2002) 767–775, https://doi.org/10.1095/biolreprod.102.004481.
[39] H. Sagirkaya, M. Misirlioglu, A. Kaya, N.L. First, J.J. Parrish, E. Memili, Developmental and molecular correlates of bovine preimplantation embryos, Reproduction 131 (2006) 895–904, https://doi.org/10.1530/rep.1.01021.
[40] S. Sugimura, T. Akai, Y. Hashiyada, T. Somfai, Y. Inaba, M. Hirayama, T. Yamanouchi, H. Matsuda, S. Kobayashi, Y. Aikawa, M. Ohtake, E. Kobayashi, K. Konishi, K. Imai, Promising system for selecting healthy in vitro-fertilized embryos in cattle, PLoS One 7 (2012) e36627, https://doi.org/10.1371/journal.pone.0036627.
[41] K. Hardy, S. Spanos, Growth factor expression and function in the human and mouse preimplantation embryo, J. Endocrinol. 172 (2002) 221–236, https://doi.org/10.1677/joe.0.1720221.
[42] H. Abe, H. Hoshi, Bovine oviductal epithelial cells: their cell culture and applications in studies for reproductive biology, Cytotechnology 23 (1997) 171–183, https://doi.org/10.1023/A:1007929826186.
[43] A.A. Ali, J.F. Bilodeau, M.A. Sirard, Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development, Theriogenology 59 (2003) 939–949, https://doi.org/10.1016/s0093-691x(02)01125-1.
[44] M. Takahashi, Oxidative stress and redox regulation on in vitro development of mammalian embryos, J. Reprod. Dev. 58 (2012) 1–9, https://doi.org/10.1262/jrd.11-138n.
[45] K. Kemp, K. Hares, E. Mallam, K.J. Heesom, N. Scolding, A. Wilkins, Mesenchymal stem cell-secreted superoxide dismutase promotes cerebellar neuronal survival, J. Neurochem. 114 (2010) 1569–1580, https://doi.org/10.1111/j.1471-4159.2009.06553.x.
[46] A.I. Caplan, Why are MSCs therapeutic? New data: new insight, J. Pathol. 217 (2009) 318–324, https://doi.org/10.1002/path.2469.
[47] A. Uccelli, L. Moretta, V. Pistoia, Mesenchymal stem cells in health and disease, Nat. Rev. Immunol. 8 (2008) 726–736, https://doi.org/10.1038/nri2395.