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Cattle production by intracytoplasmic sperm injection into oocytes vitrified after ovum pick-up

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Abstract

Intracytoplasmic sperm injection (ICSI), oocyte vitrification after ovum pick-up (OPU), and *in vitro* maturation are reproductive technologies with incredible potential for efficient cattle production. However, the developmental competence of embryos produced by ICSI using vitrified OPU oocytes remains unknown. Here, we aimed to evaluate the developmental competence of these embryos from the early embryo period to full term. The cleavage rate in the ICSI embryos using vitrified OPU oocytes during *in vitro* culture was significantly lower than those in control *in vitro* fertilized (IVF) embryos using fresh OPU oocytes (30.9 ± 4.5 % v.s. 65.9 ± 7.0%) ($P < 0.05$), but the proportion of blastocysts to cleaved embryos was significantly higher than those of IVF embryos using vitrified OPU oocytes (55.9 ± 10.8 % v.s. 23.2 ± 9.3%) ($P < 0.05$). To further investigate the transcription levels of genes related to cell differentiation in ICSI embryos using vitrified OPU oocytes, the relative abundance of mRNAs (*OCT4*, *NANOG*, *SOX2*, *CDX2*, *GATA3*, and *IFNT*) was analyzed by quantitative reverse-transcription PCR. There were no significant differences in the expression levels between ICSI embryos using vitrified OPU oocytes and control IVF embryos. Finally, developmental competence to term in ICSI embryos using vitrified OPU oocytes was examined by embryo transfer, and two healthy calves were born. These findings confirmed that ICSI and vitrification decrease developmental rates *in vitro*, but both procedures can lead to full-term development of bovine embryos. These results demonstrate that ICSI embryos using vitrification OPU oocytes are viable for cattle production.

Keywords: Bovine, Full-term development, ICSI, Oocyte vitrification, Ovum pick-up

1. Introduction

A combination of ovum pick-up (OPU) and oocytes vitrification is a valuable procedure for bovine oocyte collection and storage, making oocytes from certain genetically superior females available for repeated cattle breeding [1]. The feasibility of OPU-derived oocytes for cattle production after vitrification was confirmed by producing an offspring after *in vitro* fertilization [2]. Vitrification of unfertilized oocytes allows the preservation of female genetic resources and also facilitates breeding between sires and dams that are spatially and temporally separated. Furthermore, the development of such bovine reproductive techniques is useful for the conservation of endangered *Bos* species [3]. The improvement of vitrification protocol for unfertilized bovine oocytes has been attempted for a long time [4,5]; however, bovine embryos derived from vitrified oocytes have still shown lower developmental rates compared to embryos derived from fresh oocytes [4,5].

The vitrification process, including cryoprotectant treatment, induces premature cortical granule exocytosis, resulting in zona pellucida hardening and impairment of sperm penetration [6–8]; the denaturation of the zona pellucida is also responsible for polyspermy [8,9]. Therefore, intracytoplasmic sperm injection (ICSI) may resolve these problems, in which a single spermatozoon is directly injected into the ooplasm to bypass the zona pellucida and oolemma penetration by the spermatozoon per se. The application of ICSI to vitrified oocytes has been suggested to be more suitable than *in vitro* fertilization (IVF) for embryo production in the previous studies [10–13]. However, the developmental rates of bovine ICSI embryos using fresh oocytes have been lower than that of IVF embryos [14,15]. Additionally, offspring derived from ICSI embryos using vitrified oocytes have never been obtained, of whose cell number of the inner cell mass (ICM) was decreased compared to those of embryos using fresh oocytes [11]. Therefore, the developmental competence of ICSI embryos derived from vitrified OPU oocytes remains unknown.

In this study, we first evaluated the *in vitro* developmental competence of ICSI

76 embryos using vitrified OPU oocytes by comparing them with that of the IVF embryos using
77 fresh or vitrified OPU oocytes. Next, to confirm the integrity of ICM/trophectoderm (TE) cell
78 differentiation in ICSI blastocysts, we investigated the transcriptional levels of representative
79 cell differentiation-associated genes in ICSI embryos derived from vitrified OPU oocytes.
80 Finally, the full-term development of ICSI embryos from vitrified OPU oocytes was examined
81 by embryo transfer.

2. Materials and Methods

2.1. Animal care

All animal care and animal use in this study followed the Regulations for Animal Experiments at the Aomori Prefectural Industrial Technology Research Center.

2.2. Experimental group

Three groups were allocated to compare *in vitro* developmental competence. Each group was delineated as follows; fresh IVF group, the not vitrified fresh oocytes were fertilized using *in vitro* fertilization (IVF). In vitrified IVF group, the vitrified oocytes were fertilized using IVF. In vitrified ICSI group, the vitrified oocytes were fertilized by ICSI procedure. Investigation of the transcription levels in blastocysts by quantitative reverse-transcription PCR were performed with two groups of fresh IVF and vitrified ICSI. Only vitrified ICSI group were transferred to examine the developmental competence to term.

2.3. Ovum pick-up and oocyte in vitro maturation (IVM)

Immature oocytes were collected by ovum pick-up from Japanese Black cows. Ovarian follicles were transvaginally aspirated using a 17-gauge, 490-mm needle (Misawa Medical Industry, Ibaraki, Japan) under ultrasound guidance (HS-2200V, Honda Electronics, Aichi, Japan). Follicular contents were aspirated into a tube containing Medium 199 (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10 units/ mL heparin (Mochida Pharmaceutical, Tokyo, Japan) and 2% (v/v) newborn calf serum (NBCS; Thermo Fisher Scientific, Waltham, MA) at a constant aspiration pressure of 100 mmHg. Cumulus-oocyte complexes (COCs) were collected from the follicular contents after removing blood components using an embryo collector (Fujihira Industry, Tokyo, Japan). The collected COCs were washed and matured in 100 μL droplets of Medium 199 (M199; Thermo Fisher Scientific) supplemented with 10% (v/v) NBCS, 0.01 A.U./mL FSH (Antrin R·10; Kyoritsu Seiyaku, Tokyo, Japan), and 0.5 μg/mL

β-estradiol (Sigma Aldrich) covered with liquid paraffin (Nacalai Tesque, Kyoto, Japan) at 38.5 °C in a 5% CO_2 atmosphere for 22 h.

2.4. Oocyte vitrification and warming

After IVM culture, COCs were placed in Dulbecco's Phosphate Buffered Saline containing 300 unit/mL hyaluronidase and partially dispersed the expanded cumulus cells. Presumptively mature oocytes with some layers of cumulus cells were subjected to pre-equilibration in M199 supplemented with 3% (v/v) ethylene glycol and 20% (v/v) NBCS for 5 min. Subsequently, 5-8 oocytes were transferred to VS14 vitrification solution, composed of 5.5 M ethylene glycol, 1.0 M sucrose, and 20% (v/v) NBCS [16] for 30 s. Oocytes were then placed onto the tip of a Cryotop device (Cryotop-AG; Kitazato, Shizuoka, Japan) with a minimum quantity of vitrification solution and immediately immersed in liquid nitrogen.

The vitrified mature oocytes were warmed by directly immersing the tip of the Cryotop device into a solution consisting of M199 containing 0.5 M sucrose and 20% (v/v) NBCS at 37 °C. Retrieved oocytes were incubated for 5 min, followed by another 5-min incubation in 0.25 M sucrose solution. Oocytes were then transferred into the medium for IVM until IVF. Oocytes used for ICSI had the cumulus cells removed from the surface of the zona pellucida by gentle pipetting before ICSI. We confirmed that 89.6 ± 7.0% of the vitrified OPU oocytes were with the first polar body extrusion showing that those were presumptively at the metaphase stage of the second meiosis. Oocytes without vitrification and warming treatments were used as fresh controls for *in vitro* fertilization.

2.5. Intracytoplasmic sperm injection (ICSI)

Frozen semen from a Japanese black bull was thawed in a 37 °C water bath for 40 s, and washed twice with Bracket and Oliphant medium (BO medium) [17]. Motile spermatozoa were sorted using a microfluidic device (Sperm Sorter Qualis; Menicon Life Science, Aichi,

Japan) for 10 min, followed by dilution in 7% (v/v) polyvinylpyrrolidone (Nacalai Tesque). ICSI was performed using a piezo-driven micromanipulator (PMM-150; Prime Tech, Ibaraki, Japan) mounted on an inverted microscope (IX70; Olympus, Tokyo, Japan). Each spermatozoon was immobilized by breaking its tail and aspirated tail-first into the injection pipette. The injection pipette was transferred to a droplet containing oocyte, and the oocyte was held with a holding pipette, locating the polar body at the vertical position. The zona pellucida was penetrated by applying several piezo pulses. After ejecting the zona plug, the injection pipette was pushed forward until its tip reached the opposite side of the oocyte, and the oolemma was punctured using a single piezo pulse. The injection pipette was gently withdrawn after the spermatozoon was injected into the ooplasm. After sperm injection, the oocytes were incubated in the medium for *in vitro* maturation. Four hours after ICSI, sperm-injected oocytes were chemically activated by incubating M199 supplemented with 7% (v/v) ethanol for 5 min. After washing twice with a medium for *in vitro* culture, sperm-injected oocytes were cultured in a medium for *in vitro* culture.

2.6. In vitro fertilization (IVF)

IVF for fresh or vitrified oocytes was performed using frozen semen identical to that used for ICSI. The sperm washing media and IVF were obtained from the insemination media G-set (IVF110S; Research Institute for the Functional Peptides, Yamagata, Japan). In a water bath, frozen semen was thawed at 37 °C for 40 s. After thawing, spermatozoa were washed by suspending them in the spermatozoa washing medium of G-set and centrifuged at 2,000 rpm for 5 min. The pellet was rewashed with the insemination medium, followed by resuspension in the same medium at a 1.0×10^7/mL final concentration. COCs matured *in vitro* were washed with the insemination medium and incubated in 100 μL droplets of sperm-suspended insemination medium for 6 h at 38.5 °C in a 5% CO_2 atmosphere. After insemination, the cumulus cells of the COCs were removed by pipetting.

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161 *2.7. In vitro culture (IVC) of embryos and embryo transfer*

162 IVC for embryos was performed in a modified KSOM/aa supplemented with 10%
163 (v/v) RD medium (RPMI1640 and Dulbecco's MEM, 1:1 v/v) [18], and 1 mg/mL
164 polyvinylpyrrolidone in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C
165 under a layer of paraffin liquid. After IVF or ICSI, presumptive zygotes were washed and
166 cultured up to the blastocyst stage.

167 The four sessions of embryo transfer were performed, in which one embryo was
168 transferred to each recipient. Blastocysts from the vitrified-ICSI procedure were re-
169 cryopreserved until transfer to the recipients. Briefly, blastocysts were equilibrated for 15 min
170 in M199 medium supplemented with 20% (v/v) NBCS, 10% (v/v) ethylene glycol, 0.1 M
171 trehalose, and after that loaded into cryopreservation straw. The straws were placed in a
172 precooling programmable freezer (ET-1; Fujihira Industry) at –7 °C, and seeding was
173 performed. The cooling program was as follows; −7 °C for 10 min, −0.3 °C/min to −30 °C. The
174 straws were immediately immersed into liquid nitrogen for storage after the cooling process.

175 One cryopreserved blastocyst per recipient was thawed and transferred non-surgically
176 to a synchronized recipient of Holstein heifers. For estrous synchronization, recipient cows
177 were treated using an intravaginal progesterone-releasing device (CIDR; InterAg, Hamilton,
178 New Zealand) for 7 days with 0.5 mg cloprostenol (ZenoadinC 2 mL im; Nippon Zenyaku,
179 Fukushima, Japan) at the times of insertion. After the CIDR was withdrawn, ovulation was
180 confirmed by ultrasonography. Embryo transfer was taken place at 7 days after ovulation.

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182 *2.8. Quantitative RT-PCR analysis*

183 IVF and ICSI blastocysts were preserved in RNA*later* Solution (Thermo Fisher
184 Scientific) at -20 °C until RNA extraction. Total RNA from five blastocysts per biological
185 replicate was isolated using ReliaPrep RNA Cell Miniprep System (Promega, Fitchburg, WI,

USA). Reverse transcription from RNA was performed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Five biological replicates were analyzed for each group. Quantitative PCR (qPCR) was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) and the specified primers (Table 1). Thermal cycling conditions consisted of 1 cycle at 95 °C for 30 s, followed by 45 cycles at 95 °C for 10 s, annealing temperature corresponding to each primer set for 10 s, and 72 °C for 30 s. Relative mRNA abundance was calculated using the ΔΔCt method, where *H2A histone family member Z* (*H2AFZ*) was used as a reference gene for each sample. All quantitative RT-PCR analyses were performed in triplicates.

2.9. Statistical analysis

The proportional data for *in vitro* development were analyzed by one-way ANOVA after arcsine-transformation and Bartlett's test for homogeneity of variance. A comparison of values among the different groups was performed using Tukey-Kramer multiple comparison tests. The mRNA expression levels were assessed by Student's *t*-test based on the $2^{-\Delta\Delta Ct}$ values. Differences were considered significant when $P < 0.05$.

3. Results

3.1. In vitro *developmental competence of ICSI embryos derived from vitrified OPU oocytes*

In vitro developmental rates of vitrified ICSI group were compared with those of fresh IVF and vitrified IVF group (Table 2). The cleavage rate of ICSI embryos (30.9 ± 4.5%) was significantly lower than that of fresh IVF group (65.9 ± 7.0%) ($P < 0.05$). Nevertheless, there was no significant difference in the blastocyst formation rate between vitrified ICSI group (16.5 ± 7.0%) and fresh IVF group (35.3 ± 4.2%). The blastocyst formation rate of vitrified IVF group (12.8 ± 4.5%) was significantly lower than that of fresh IVF group ($P < 0.05$). The proportions of blastocysts to cleaved embryos in ICSI embryos (55.9 ± 10.8%) was significantly higher than that in vitrified IVF embryos (23.2 ± 9.3%) ($P < 0.05$). These results show that oocyte vitrification impairs embryonic development *in vitro* in IVF and ICSI procedures.

3.2. Gene expression of differentiation markers in ICSI blastocysts derived from vitrified OPU oocytes

To assess the effect of both vitrification and ICSI procedures on the cell differentiation to ICM and TE lineages at the blastocyst stage, the relative expression levels of six representative differentiation-associated genes (*OCT4*, *SOX2*, *NANOG*, *CDX2*, *GATA3*, and *IFNT*) were determined by qPCR (Fig. 1). The expression level of vitrified ICSI group blastocysts was compared with fresh IVF embryos. There were no significant differences in mRNA expression levels of all analyzed genes between the ICSI and IVF embryos, suggesting that both vitrification and ICSI procedures do not affect the expression of differentiation-associated genes at the blastocyst stage.

3.3. Full-term development of ICSI blastocysts derived from vitrified OPU oocytes

Four blastocysts were transferred to four surrogate heifers to validate that ICSI embryos using vitrified OPU oocytes could develop to term (Table 3). Among them, three

recipients were pregnant, and two healthy calves were born with regular body weights (33.0 kg and 29.3 kg) after the average gestational period (Fig. 2).

4. Discussion

In this study, we applied the ICSI procedure to bovine oocytes, which were retrieved by OPU and vitrified after IVM, and consequently, we succeeded in producing viable calves by transferring ICSI embryos to recipients. Therefore, we demonstrated that ICSI using vitrified OPU oocytes is a feasible strategy for cattle production. Although the ICSI embryos showed a lower cleavage rate than those of IVF embryos using fresh oocytes, the proportion of blastocysts to cleaved embryos in the ICSI embryos was comparable to those of IVF embryos using fresh oocytes. At the same time, it was significantly higher than IVF embryos using vitrified oocytes. These findings were consistent with the previous other studies [11,13]. ICSI procedure might improve developmental rate after cleavage-stage due to avoidance of the fertilization failure such as polyspermy caused by the vitrification process [8,9].

Since the proportion of blastocysts to cleaved embryos was comparable between vitrified ICSI group and fresh IVF group in this study, the low developmental competence of ICSI embryos can be ascribed to limited occurrence of cleavage in ICSI protocol. Unlike other species, early fertilization events after sperm injection in bovine oocytes can be defective due to several unique attributes of this species [19,20]. Therefore, further improvement of ICSI protocol in cattle would be required for enhancement of the cleavage rate in vitrified ICSI embryos. Improvement of *in vitro* maturation conditions or development of effective methods that induce natural Ca^{2+} oscillation [21–23] or decondensation of spermatozoa [21,24,25] after injection may improve the developmental efficiency after ICSI in cattle.

In comparing gene expression relating to cell differentiation at the blastocyst stage, there were no significant differences of all analyzed six genes (*OCT4*, *NANOG*, *SOX2*, *CDX2*, *GATA3*, and *IFNT*) between the ICSI and IVF embryos. These six genes play essential roles in bovine early embryogenesis. *OCT4* is required for blastocyst formation [26]. Both *NANOG* and *SOX2* are well known as pluripotency factors [27,28]. Meanwhile, *CDX2* and *GATA3* are required for TE lineage establishment [29]. In the knockdown approach using RNA interference,

OCT4 and *CDX2* downregulation in bovine embryos impair the blastocyst development and the expression of *GATA3* and *NANOG* [30,31]. *IFNT* is not strictly involved in the ICM/TE segregation but is a pregnancy recognition factor in ruminant animals [32]. *IFNT* is explicitly expressed in TE cells under the regulation of *GATA3*, whose expression level indicates usually differentiated TE cells [33]. Therefore, we chose these six genes as representative cell differentiation markers to evaluate the effect of vitrification and ICSI procedures on appropriate cell differentiation during blastocyst formation. Consequently, we did not detect any differences in these gene expression levels between the vitrified ICSI blastocysts and the fresh IVF blastocysts, suggesting that both vitrification and ICSI procedures do not affect cell differentiation during blastocyst formation *in vitro*.

From the perspective of artificial reproductive technology, the results demonstrated in this study provide an important model for animal conservation. Development of the biotechnologies including germplasm storage like as oocyte cryopreservation would be critical for allowing their use as a tool for animal conservation such as endangered species [3]. In addition, ICSI can be a useful technique for artificial fertilization instead of establishing the IVF conditions which optimized for each species. The strategy to reproduce using oocyte vitrification coincides with One Conservation concept, which is proposed recently, and defined as comprehensive interconnection between *in situ* and *ex situ* conservation plans, actions and researches in different areas that encompass conservation [34]. Oocyte vitrification and full-term development of bovine using ICSI in this study can be extrapolated to the endangered *Bos* species, such as wild yak (*Bos mutus*), gaur (*Bos gaurus*), banteng (*Bos javanicus*) and kouprey (*Bos sauveli*).

The present study showed that ICSI embryos using bovine vitrified OPU oocytes that developed up to the blastocyst stage *in vitro* had standard developmental competency to full-term. Therefore, further technical improvement, including optimization of *in vitro* maturation, oocyte vitrification, and sperm treatment, is necessary to make embryo production by ICSI

using vitrified OPU oocytes more reliable.

5. Conclusions

The present study showed that the application of ICSI to vitrified OPU oocytes. Although embryo production efficiency is still limited compared to those using fresh oocytes, the gene expression analysis of cell differentiation and full-term development after embryo transfer demonstrated that ICSI and vitrification procedures do not prevent developmental competence to term. Thus, these results demonstrate feasibility of cattle production, suggesting important model for assisted reproductive technologies for conservation of endangered animal.

Declaration of interest

The authors declare no conflicts of interest.

Author Contributions

S.K. and M.K. designed the experiments. S.K. conducted the experiments. S.K. and M.K. analyzed the data. H.B. and M.T. provided support in the preparation of the materials for the experiments. S.K. and M.K. drafted the manuscript. S.H. conceptualized the project. All authors contributed to the interpretation of the data and read and approved the final manuscript.

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Figure captions

Figure 1. Comparison of relative mRNA expression levels of genes related to cell differentiation in blastocyst stage embryos between different fertilized methods. White bars: IVF blastocysts using fresh oocytes. Black bars: ICSI blastocysts using vitrified oocytes. Values are represented as means ± SEM (indicated by error bars). The NS. abbreviation shows the not significant differences. Levels of any analyzed genes did not differ significantly in each group.

Figure 2. Two calves were derived from ICSI embryos using vitrified OPU oocytes. (Left): A female calf whose body weight was 33.0 kg, was born 278 days after embryo transfer. (Right): A male calf whose body weight was 29.3 kg, was born 288 days after embryo transfer.

Highlights

• ICSI using vitrified OPU bovine oocytes impairs the cleavage rate in *in vitro* culture.

• Procedures of ICSI and vitrification do not affect the cell differentiation gene expression.

• ICSI embryos derived from vitrified OPU oocytes can develop to term.

Table 1

Primer sets for the quantitative RT-PCR analysis.

Gene	Accession number	Primer sequence (5'-3')	Annealing temperature	Product size (bp)
OCT4	NM_174580.3	F: ACATGTGTAAGCTGCGGCCC	58	107
		R: CTTTCGGGCCTGCACAAGGG		
NANOG	NM_001025344.1	F: CTCGCAGACCCAGCTGTGTG	58	198
		R: CCCTGAGGCATGCCATTGCT		
SOX2	NM_001105463.2	F: GCAGACCTACATGAACGGCT	58	245
		R: ACATGTGAAGTCTGCTGGGG		
CDX2	NM_001206299.1	F: GCCACCATGTACGTGAGCTAC	58	140
		R: ACATGGTATCCGCCGTAGTC		
GATA3	NM_001076804.1	F: CTACCACAAGATGAACGGACAG	58	142
		R: AGGGTCTCCATTGGCATTTC		
IFNT	NM_001031765.1	F: CTACTGATGGCCTTGGTGCT	55	204
		R: GTCCTTCTGGAGCTGGTCAC		
H2AFZ	NM_174809.2	F: AGAGCCGGTTTGCAGTTCCCG	58	116
		R: TACTCCAGGATGGCTGCGCTGT		

Table 2

In vitro development of bovine ICSI embryos using vitrified OPU oocytes and IVF embryos using fresh or vitrified oocytes.

Group	No. of replicates	No. of oocytes	No. (%) of cleaved[†]	No. (%) of blastocyst[†]	Blastocysts / cleaved embryos (%)
fresh - IVF	6	98	68 (65.9 ± 7.0) [a]	34 (35.3 ± 4.2) [a]	55.3 ± 6.3 [ab]
vitrified - IVF	8	132	69 (53.2 ± 9.0) [ab]	18 (12.8 ± 4.5) [b]	23.2 ± 9.3 [a]
vitrified - ICSI	8	140	43 (30.9 ± 4.5) [b]	22 (16.5 ± 3.5) [ab]	55.9 ± 10.8 [b]

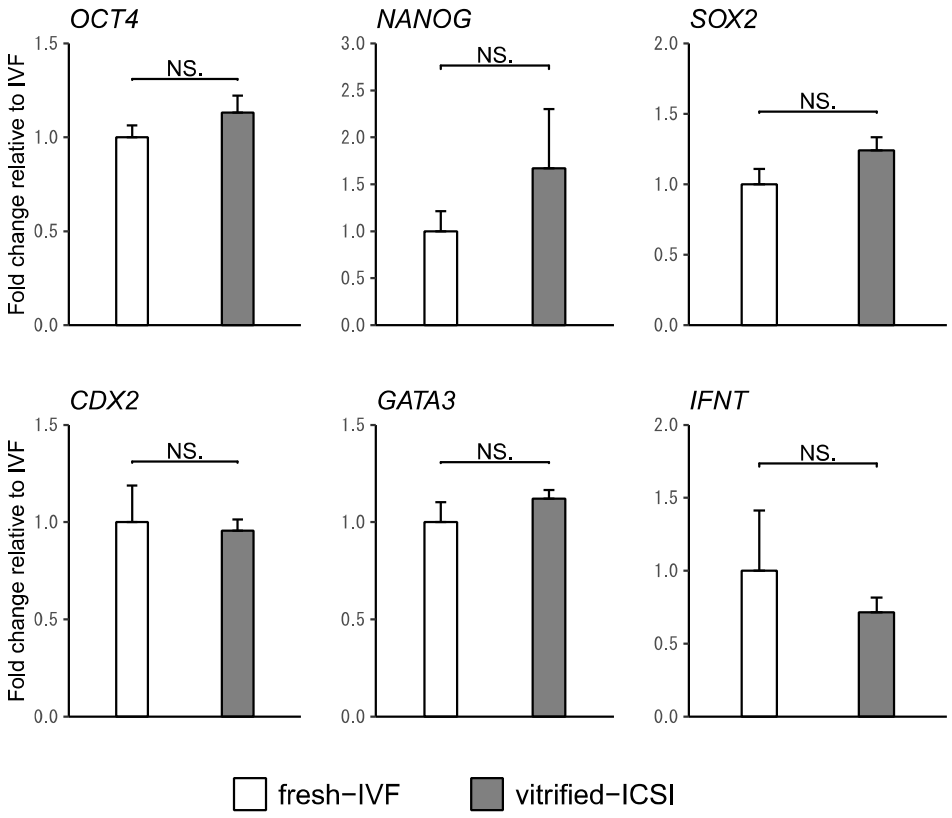
[†]These percentages were calculated using the number of used oocytes that are shown in the left column. Values are shown as the mean ± SEM. Different superscript letters (a, b) within the same column indicate significantly different values ($P < 0.05$).

Table 3

The number of pregnancy and calves to term by transferring the ICSI blastocysts using vitrified OPU oocytes.

No. of transferred recipients	No. of pregnant cows (%)	No. of abortions	No. of calves to term
4	3 (75.0)	1	2

One embryo was transferred to each cow.



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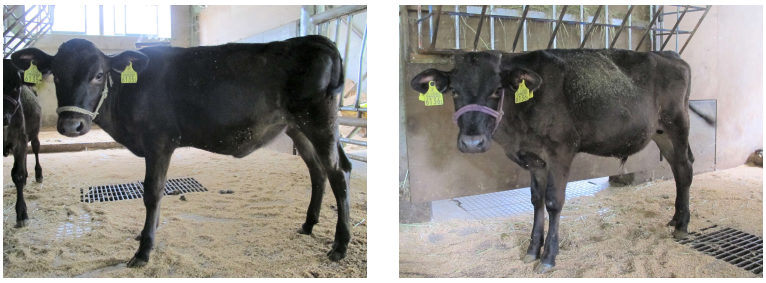

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