



Title	Cattle production by intracytoplasmic sperm injection into oocytes vitrified after ovum pick-up
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1 **Cattle production by intracytoplasmic sperm injection into oocytes vitrified after ovum**  
2 **pick-up**

3

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26

27 **Abstract**

28 Intracytoplasmic sperm injection (ICSI), oocyte vitrification after ovum pick-up (OPU), and *in*  
29 *vitro* maturation are reproductive technologies with incredible potential for efficient cattle  
30 production. However, the developmental competence of embryos produced by ICSI using  
31 vitrified OPU oocytes remains unknown. Here, we aimed to evaluate the developmental  
32 competence of these embryos from the early embryo period to full term. The cleavage rate in  
33 the ICSI embryos using vitrified OPU oocytes during *in vitro* culture was significantly lower  
34 than those in control *in vitro* fertilized (IVF) embryos using fresh OPU oocytes ( $30.9 \pm 4.5\%$   
35 v.s.  $65.9 \pm 7.0\%$ ) ( $P < 0.05$ ), but the proportion of blastocysts to cleaved embryos was  
36 significantly higher than those of IVF embryos using vitrified OPU oocytes ( $55.9 \pm 10.8\%$  v.s.  
37  $23.2 \pm 9.3\%$ ) ( $P < 0.05$ ). To further investigate the transcription levels of genes related to cell  
38 differentiation in ICSI embryos using vitrified OPU oocytes, the relative abundance of mRNAs  
39 (*OCT4*, *NANOG*, *SOX2*, *CDX2*, *GATA3*, and *IFNT*) was analyzed by quantitative reverse-  
40 transcription PCR. There were no significant differences in the expression levels between ICSI  
41 embryos using vitrified OPU oocytes and control IVF embryos. Finally, developmental  
42 competence to term in ICSI embryos using vitrified OPU oocytes was examined by embryo  
43 transfer, and two healthy calves were born. These findings confirmed that ICSI and vitrification  
44 decrease developmental rates *in vitro*, but both procedures can lead to full-term development of  
45 bovine embryos. These results demonstrate that ICSI embryos using vitrification OPU oocytes  
46 are viable for cattle production.

47

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

49

## 50 **1. Introduction**

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

62 The vitrification process, including cryoprotectant treatment, induces premature  
63 cortical granule exocytosis, resulting in zona pellucida hardening and impairment of sperm  
64 penetration [6–8]; the denaturation of the zona pellucida is also responsible for polyspermy  
65 [8,9]. Therefore, intracytoplasmic sperm injection (ICSI) may resolve these problems, in which  
66 a single spermatozoon is directly injected into the ooplasm to bypass the zona pellucida and  
67 oolemma penetration by the spermatozoon per se. The application of ICSI to vitrified oocytes  
68 has been suggested to be more suitable than *in vitro* fertilization (IVF) for embryo production  
69 in the previous studies [10–13]. However, the developmental rates of bovine ICSI embryos  
70 using fresh oocytes have been lower than that of IVF embryos [14,15]. Additionally, offspring  
71 derived from ICSI embryos using vitrified oocytes have never been obtained, of whose cell  
72 number of the inner cell mass (ICM) was decreased compared to those of embryos using fresh  
73 oocytes [11]. Therefore, the developmental competence of ICSI embryos derived from vitrified  
74 OPU oocytes remains unknown.

75 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.

## 82 **2. Materials and Methods**

### 83 *2.1. Animal care*

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

86

### 87 *2.2. Experimental group*

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

95

### 96 *2.3. Ovum pick-up and oocyte in vitro maturation (IVM)*

97 Immature oocytes were collected by ovum pick-up from Japanese Black cows. Ovarian  
98 follicles were transvaginally aspirated using a 17-gauge, 490-mm needle (Misawa Medical  
99 Industry, Ibaraki, Japan) under ultrasound guidance (HS-2200V, Honda Electronics, Aichi,  
100 Japan). Follicular contents were aspirated into a tube containing Medium 199 (Sigma Aldrich,  
101 St. Louis, MO, USA) supplemented with 10 units/ mL heparin (Mochida Pharmaceutical, Tokyo,  
102 Japan) and 2% (v/v) newborn calf serum (NBCS; Thermo Fisher Scientific, Waltham, MA) at  
103 a constant aspiration pressure of 100 mmHg. Cumulus-oocyte complexes (COCs) were  
104 collected from the follicular contents after removing blood components using an embryo  
105 collector (Fujihira Industry, Tokyo, Japan). The collected COCs were washed and matured in  
106 100  $\mu$ L droplets of Medium 199 (M199; Thermo Fisher Scientific) supplemented with 10%  
107 (v/v) NBCS, 0.01 A.U./mL FSH (Antrin R-10; Kyoritsu Seiyaku, Tokyo, Japan), and 0.5  $\mu$ g/mL

108  $\beta$ -estradiol (Sigma Aldrich) covered with liquid paraffin (Nacalai Tesque, Kyoto, Japan) at  
109 38.5 °C in a 5% CO<sub>2</sub> atmosphere for 22 h.

110

#### 111 2.4. Oocyte vitrification and warming

112 After IVM culture, COCs were placed in Dulbecco's Phosphate Buffered Saline  
113 containing 300 unit/mL hyaluronidase and partially dispersed the expanded cumulus cells.  
114 Presumptively mature oocytes with some layers of cumulus cells were subjected to pre-  
115 equilibration in M199 supplemented with 3% (v/v) ethylene glycol and 20% (v/v) NBCS for 5  
116 min. Subsequently, 5-8 oocytes were transferred to VS14 vitrification solution, composed of  
117 5.5 M ethylene glycol, 1.0 M sucrose, and 20% (v/v) NBCS [16] for 30 s. Oocytes were then  
118 placed onto the tip of a Cryotop device (Cryotop-AG; Kitazato, Shizuoka, Japan) with a  
119 minimum quantity of vitrification solution and immediately immersed in liquid nitrogen.

120 The vitrified mature oocytes were warmed by directly immersing the tip of the Cryotop  
121 device into a solution consisting of M199 containing 0.5 M sucrose and 20% (v/v) NBCS at  
122 37 °C. Retrieved oocytes were incubated for 5 min, followed by another 5-min incubation in  
123 0.25 M sucrose solution. Oocytes were then transferred into the medium for IVM until IVF.  
124 Oocytes used for ICSI had the cumulus cells removed from the surface of the zona pellucida by  
125 gentle pipetting before ICSI. We confirmed that  $89.6 \pm 7.0\%$  of the vitrified OPU oocytes were  
126 with the first polar body extrusion showing that those were presumptively at the metaphase  
127 stage of the second meiosis. Oocytes without vitrification and warming treatments were used  
128 as fresh controls for *in vitro* fertilization.

129

#### 130 2.5. Intracytoplasmic sperm injection (ICSI)

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

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

148

## 149 2.6. *In vitro* fertilization (IVF)

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



160

## 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<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> 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.

181

## 182 *2.8. Quantitative RT-PCR analysis*

183 IVF and ICSI blastocysts were preserved in RNAlater 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,

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

194

### 195 2.9. Statistical analysis

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

201

202 **3. Results**

203 *3.1. In vitro developmental competence of ICSI embryos derived from vitrified OPU oocytes*

204 *In vitro* developmental rates of vitrified ICSI group were compared with those of fresh  
205 IVF and vitrified IVF group (Table 2). The cleavage rate of ICSI embryos ( $30.9 \pm 4.5\%$ ) was  
206 significantly lower than that of fresh IVF group ( $65.9 \pm 7.0\%$ ) ( $P < 0.05$ ). Nevertheless, there  
207 was no significant difference in the blastocyst formation rate between vitrified ICSI group ( $16.5$   
208  $\pm 7.0\%$ ) and fresh IVF group ( $35.3 \pm 4.2\%$ ). The blastocyst formation rate of vitrified IVF group  
209 ( $12.8 \pm 4.5\%$ ) was significantly lower than that of fresh IVF group ( $P < 0.05$ ). The proportions  
210 of blastocysts to cleaved embryos in ICSI embryos ( $55.9 \pm 10.8\%$ ) was significantly higher  
211 than that in vitrified IVF embryos ( $23.2 \pm 9.3\%$ ) ( $P < 0.05$ ). These results show that oocyte  
212 vitrification impairs embryonic development *in vitro* in IVF and ICSI procedures.

213

214 *3.2. Gene expression of differentiation markers in ICSI blastocysts derived from vitrified OPU*  
215 *oocytes*

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

224

225 *3.3. Full-term development of ICSI blastocysts derived from vitrified OPU oocytes*

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

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

#### 230 4. Discussion

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

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

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

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

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

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

282 using vitrified OPU oocytes more reliable.

283

## 284 **5. Conclusions**

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

291

## 292 **Declaration of interest**

293           The authors declare no conflicts of interest.

294

## 295 **Author Contributions**

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

300

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306

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422 **Figure captions**

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

428

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

432

## 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)
<i>OCT4</i>	NM_174580.3	F: ACATGTGTAAGCTGCGGCC R: CTTTCGGGCCTGCACAAGGG	58	107
<i>NANOG</i>	NM_001025344.1	F: CTCGCAGACCCAGCTGTGTG R: CCCTGAGGCATGCCATTGCT	58	198
<i>SOX2</i>	NM_001105463.2	F: GCAGACCTACATGAACGGCT R: ACATGTGAAGTCTGCTGGGG	58	245
<i>CDX2</i>	NM_001206299.1	F: GCCACCATGTACGTGAGCTAC R: ACATGGTATCCGCCGTAGTC	58	140
<i>GATA3</i>	NM_001076804.1	F: CTACCACAAGATGAACGGACAG R: AGGGTCTCCATTGGCATTTC	58	142
<i>IFNT</i>	NM_001031765.1	F: CTA CTGATGGCCTTGGTGCT R: GTCCTTCTGGAGCTGGTCAC	55	204
<i>H2AFZ</i>	NM_174809.2	F: AGAGCCGGTTTGCAGTTCCCG R: TACTCCAGGATGGCTGCGCTGT	58	116

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

<sup>†</sup>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 ( $P < 0.05$ ).

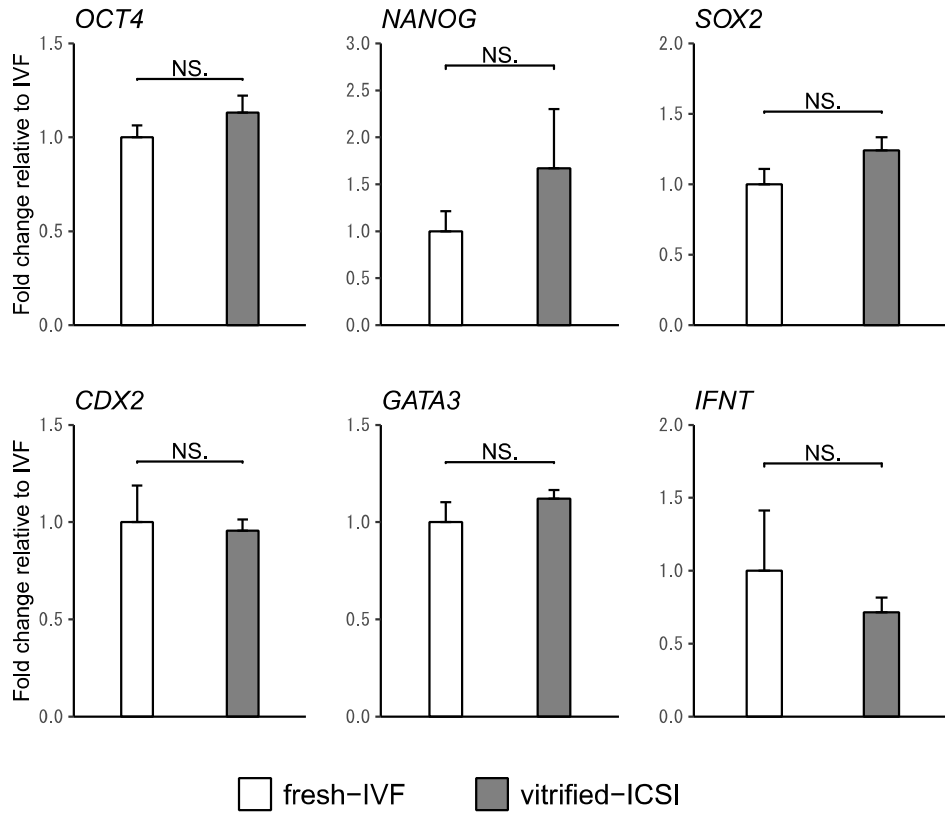


**Table 3**

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.



**Fig. 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 oocyte. Values are represented as means  $\pm$  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.



**Fig. 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.