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NOTE

Theriogenology

Production of Japanese Black calves by the transfer of embryos developed from *in vitro*-fertilized oocytes derived by ovum pick up and matured in culture with the mitogen-activated protein kinase kinase inhibitor U0126

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ABSTRACT. This study investigated whether treatment with the mitogen-activated protein kinase kinase inhibitor U0126 during *in vitro* maturation (IVM), which has previously been reported to improve oocyte developmental competence, is practical for use in calf production using ovum pick up (OPU)-derived oocytes. Two Japanese Black cows were repeatedly and simultaneously treated to stimulate follicular growth and were prepared for OPU. Cumulus-oocyte complexes (COCs) were collected from one cow using a collection medium containing 5 μM U0126 and were cultured in medium supplemented with the same concentration of U0126 for the first 2 hr of IVM; COCs from the other cow were used as controls without U0126 treatment. The cows were exchanged between the two groups at every sequential OPU (n=8). The number of oocytes developing to blastocysts in the U0126-treated group (39.1%, 34/87) was significantly higher than that in the control group (22.1%, 19/86). Eight blastocysts produced with U0126 treatment were transferred to recipients, and four normal calves were obtained. The results indicate that embryos develop efficiently from OPU-derived oocytes treated with U0126, and that these embryos may be of practical use in calf production.

KEY WORDS: cattle, *in vitro* maturation, MAPK kinase, OPU, U0126

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Following a surge in blood luteinizing hormone (LH) concentrations, mitogen-activated protein kinase (MAPK) becomes activated and causes a breakdown in the gap junctions (GAPJs) between oocytes and cumulus cells. This in turn causes a decrease in oocyte cAMP levels and the resumption of meiosis [4]. Previous reports have shown that the rate at which *in vivo*-matured oocytes develop into blastocysts is higher than that for *in vitro*-matured oocytes [2, 5]. Although the reason for this is not clear, gonadotropins and growth factors are added to the culture medium of *in vitro*-matured oocytes [5, 10], which might activate MAPK via MAPK kinase, leading to the rapid breakdown of GAPJs and a resumption of meiosis even though there is insufficient cytoplasmic maturation. In order to efficiently produce embryos using the ovum pick up (OPU) method, it is therefore desirable to use *in vivo*-matured oocytes. However, the collection of *in vivo*-matured oocytes gradually becomes more difficult due to declining ovarian responses to repeated gonadotropin treatments, and the fact that estrous cows bearing *in vivo*-matured oocytes are nervous and difficult to manage. Furthermore, it is difficult to collect the cumulus oocyte complexes (COCs) with mature oocytes, because they have swollen cumulus cells. One obstacle to the use of *in vitro*-matured oocytes for efficient calf production is the low production rate of transferable embryos. Shinohara *et al*. [8] have reported that treatment with the MAPK kinase inhibitor U0126 during the first 2 hr of *in vitro* maturation (IVM) delays the breakdown of GAPJs between the oocytes and the cumulus cells and improves bovine oocyte developmental competence. However, in this latter study the COCs were derived from ovaries obtained at a slaughterhouse. Therefore, this study examined whether treatment with U0126 could be used in practice for calf production using OPU-derived oocytes in Japanese Black cows.

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Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). IVM and *in vitro* fertilization (IVF) were performed using IVMD101 and IVF100 media (Research Institute for the Functional Peptides, Yamagata, Japan), respectively. The media constituents have previously been reported by Yamashita *et al*. [10]. All cultures used chemically defined and serum-free media. The animal experiments in this study were approved by the Institutional Animal Experiment Committee of Kanagawa Prefectural Livestock Industry Technology Center. Two Japanese Black cows were repeatedly used for OPU. Controlled intravaginal progesterone releasing devices (CIDR; Zoetis Japan, Tokyo, Japan) were inserted into the vagina on any day of the estrous cycle except during the estrous stage. At the same time, the cows were injected subcutaneously with 20 AU of porcine follicle stimulating hormone (pFSH, Antrin-R10; Kyoritsu Seiyaku Corp., Tokyo, Japan) dissolved in 50 m*l* of physiological saline, as described by Hiraizumi *et al*. [3]. The CIDRs were removed 72 hr after the injection of pFSH, and the follicles were aspirated using the OPU method described by Sakagami *et al.* [6]. Briefly, an ultrasound scanner (ECHOPAL II, Hitachi Medical, Tokyo, Japan) with a 6.5-MHz probe and a disposable needle (COVA Needle, Misawa Medical Industry, Tokyo, Japan) were inserted into the vagina of each Japanese Black cow, and the number of follicles over 2 mm in diameter was counted. The follicular contents were aspirated into a centrifuge tube containing the collection medium, which was composed of Ringer's lactate solution (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) supplemented with 10 IU/m*l* heparin and 1% bovine serum with or without 5 μM U0126 (Promega Corp., Madison, WI, U.S.A.). On average, it took 40–50 min to transfer the collected COCs from the collection medium into the IVM medium. Therefore, U0126 was added not only to the IVM medium but also to the collection medium. U0126 was dissolved in dimethyl sulfoxide (DMSO) and the control COCs were collected into collection medium containing 0.1% DMSO only. The aspirated follicular contents were filtered through an EM CON^{TM} filter (AG Japan, Suzuka, Japan). COCs that had oocytes with a homogeneous cytoplasm were used regardless of whether or not there were adherent cumulus cells. However, COCs that were swollen or had a "cobweb-like" appearance were not used even if the oocyte cytoplasm appeared normal. COCs collected from one cow were assigned to the U0126-treated group, and those from the other cow were used as controls. The individual cow assigned to each group was changed at every sequential OPU. OPU from the two cows was repeated a total of eight times. IVM, IVF, and the *in vitro* culture of embryos (IVC) were performed using a modified versions of methods described in previous reports [6–8]. The number of oocytes cultured in a drop of medium was equal to the number of oocytes selected from a cow (Table 1; the number of oocytes used). Briefly, COCs in the U0126-treated group were cultured in a 100 μ*l* droplet of IVMD101 containing 5 μM U0126 for 2 hr at 38.5°C in a humidified atmosphere of 5% CO_2 in air. The IVMD101 media used for the controls contained 0.1% DMSO and no U0126. IVM was continued for both groups by incubating the COCs in IVMD101 for 22 hr. IVF was performed using a frozen stock of semen obtained from a Japanese Black bull. The COCs were washed three times with IVF100, transferred into 100 μ*l* microdroplets containing sperm for insemination (5.0×10^6 spermatozoa/m*l*), and incubated for 6 hr at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After IVF, the cumulus cells were removed from the oocytes by pipetting, and presumptive zygotes were incubated in a 100 μ*l* drop of glucose-free modified synthetic oviduct fluid culture medium [9] supplemented with 2% (v/v) Basal Medium Eagle essential amino acids (B6766), 1% (v/v) minimum essential medium (MEM; 11140–050, Thermo Fisher Scientific KK., Tokyo, Japan), 1 mg/m*l* polyvinyl alcohol (P8136), 100 *n*g/m*l* epidermal growth factor (E4127), 50 *n*g/m*l* insulin-like growth factor I (I3769), 5 *n*g/m*l* selenium, and 5 μg/m*l* transferrin at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2. Although a previous study did not add selenium and transferrin to the culture media [6], their addition was recommended by Barnes and Sato [1]. Since they had a stimulatory effect on oocyte oxygen consumption (unpublished data), they were included in this study. Glucose was added to the culture medium at a concentration of 4 mM five days after fertilization, as described in a previous report [7], and the rate of oocytes that had undergone cleavage and development to the blastocyst stage was determined 2 and 8 days after fertilization, respectively. Blastocysts obtained from the U0126-treated group (n=8) were transferred into the uterine horn of recipients (Holstein or Japanese Black cattle) whose ovulation had been confirmed seven days earlier. Embryo transfer was performed after confirming the presence of the corpus luteum. Ovulation and presence of the corpus luteum were determined by rectal palpation. Each blastocyst was transferred to one recipient. Pregnancy was confirmed using ultrasonography 55 days after transfer. The newborn calves were weighed and checked for external abnormalities and gender. Gestation periods were also recorded.

Data are expressed as mean ± standard error of the mean (SEM). The square root of the rate of oocytes developing to blastocysts in each drop of the culture media was arcsine transformed for statistical analysis. A comparison of the values between the two groups was performed using a Student's *t*-test or Welch's *t*-test after an *F* test for homogeneity of variance. A *P* value less than 0.05 was considered statistically significant, and less than 0.1 was considered as a tendency to be different.

Table 1. Effects of U0126 treatment during oocyte collection and the first 2 hr of IVM on the developmental competence of oocytes collected from Japanese Black cows using the OPU method

Treatment	No. of follicles[a)]	No. of oocytes collected[a)]	No. of oocytes used[a)]	No. of cleaved zygotes[a)]	No. of blastocysts at day 8[a)]	Total no. of blastocysts/ total no. of oocytes used (%)[b)]
Control	22.1 ± 2.5	12.0 ± 3.6	10.8 ± 3.5	6.9 ± 3.0	2.4 ± 0.9	19/86 (22.1)
U0126	27.4 ± 4.6	12.6 ± 1.9	10.9 ± 1.9	7.5 ± 1.6	4.3 ± 0.7[#]	34/87 (39.1)*

OPU from two cows was replicated 8 times (n=8). The COCs collected from one cow were used in the group treated with U0126 and those collected from the other cow were used in the control group. The assignment of the cows to each group was exchanged at every sequential OPU. a) Values are the mean ± SEM. b) Values were statistically analyzed by the arcsin-transformed values obtained from square roots of the rates of oocytes developing to blastocysts in each drop of the culture media. [#]$P<0.1$ compared to the controls. *$P<0.05$ compared to the controls.

Table 2. Pregnancy rate, gestation period, and the number and weights of the calves obtained after the transfer of embryos developed from OPU-derived Japanese Black cow oocytes treated with U0126 during oocyte collection and the first 2 hr of IVM

No. of embryos transferred[a)]	No. of pregnant cows (%)	No. of abortions	Male calves			Female calves		
			No. of calves obtained	Body weight (kg)	Gestation period (days)	No. of calves obtained	Body weight (kg)	Gestation period (days)
8	5 (62.5)	1	3	40.5 ± 2.0[b)]	287.7 ± 0.3[b)]	1	40.9	289

a) One embryo was transferred to each cow. b) Values are the mean ± SEM.

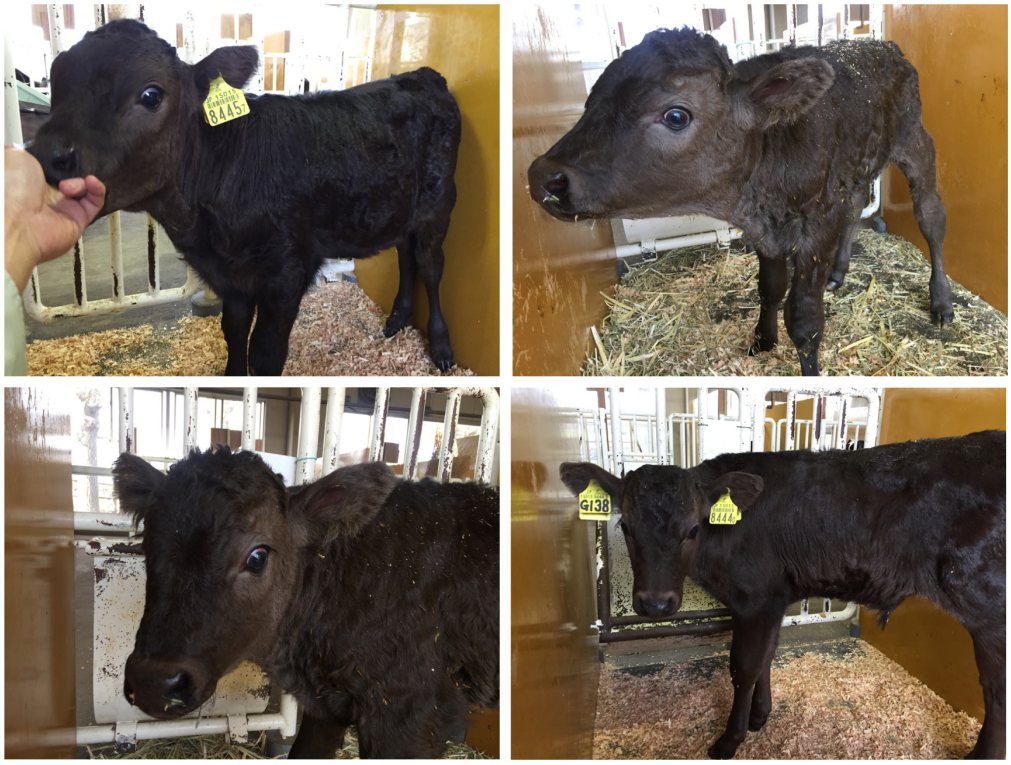

Fig. 1. One female (upper left) and three male calves were obtained after the transfer of embryos developed from OPU-derived Japanese Black cow oocytes treated with U0126 during oocyte collection and the first 2 hr of IVM.

Table 1 shows the effects of U0126 treatment on the developmental competence of oocytes collected using the OPU method. Although no differences were found in the number of follicles, collected oocytes, oocytes used, and cleaved zygotes between the different groups, the number of blastocysts in the U0126-treated group tended to be higher than that in the control group (P=0.09, Student's t-test). The rate of oocytes developing to the blastocyst stage (39.1%) in the U0126-treated group was significantly higher than that in the control group (22.1%; P=0.03, Welch's t-test). These results suggest that treatment with U0126 improves the developmental competence of OPU-derived bovine oocytes. However, it should be noted that in the present study, U0126 was added not only to the IVM medium, but also to the collection medium, since COCs collected by OPU were kept in the collection medium for a longer time than those collected from ovaries obtained at an abattoir. The effect of U0126 addition to the collection medium on oocyte developmental competence remains to be studied.

Table 2 shows the pregnancy rates, gestation periods, and numbers and weights of male and female calves after the transfer of embryos treated with U0126. The pregnancy rate was relatively high (62.5%), and abortion after confirmation of pregnancy using ultrasonography was observed in only one cow. The gestation periods of the cows fell within a narrow range between 287 and 289 days, indicating that they were not different from those of artificially inseminated Japanese Black cows in our study (mean ± SEM: 288.1 ± 4.4 days; n=122, unpublished data). One female and three male calves were born, and neither difficult calving nor abnormalities in the calves were observed (Fig. 1). Therefore, the rate of transferred embryos growing to newborn calves was 4/8 (50%). Based on our previous report on the transfer of OPU-derived embryos developed in a serum-added culture medium [6], the rates of pregnancy and embryos developing to newborn calves were 6/18 (33.3%) and 4/18 (22.2%), respectively. Although the number of transferred embryos in the previous report and in the present study were both low, it is possible that embryos that develop from OPU-derived oocytes treated with U0126 implant and develop to newborn calves without critical defects more successfully than embryos developed in the serum-added culture medium. Although the weights of the calves obtained in the

present study were relatively high, there was no significant difference (P=0.39, Student's t test) in the weight of male calves between the present study (40.5 ± 2.0 kg, n=3) and our previous work [6] that used OPU-derived oocytes cultured without U0126 during IVM and with fetal bovine serum during IVC (36.5 ± 3.5 kg, n=3). Therefore, it is possible that the effect of treatment with U0126 on fetal growth may compare favorably with that of the common culture protocols to produce transferable embryos.

In conclusion, the results of the present study indicate that bovine embryos can develop efficiently from OPU-derived oocytes treated with 5 μM U0126 during oocyte collection and the first 2 hr of IVM. Furthermore, the embryos obtained following this treatment exhibit normal growth to newborn calves after their transfer.

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