FULL PAPER *Theriogenology*

Birth Weight and Gestation Length of Japanese Black Calves Following Transfer of Embryos Produced *in Vitro* with or without Co-Culture

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ABSTRACT. Birth weight and gestation length of calves following the transfer of *in vitro* produced (IVP) embryos with or without co-culture of cumulus cells, were compared to those produced *in vivo* (IVD). Spermatozoa from one Japanese Black bull were used for both IVP and IVD. IVP embryos were produced using two types of culture method: 1) co-culturing with cumulus cells in TCM 199 supplemented with calf serum (IVP-Co), and 2) non-co-culturing without cumulus cells in CR1aa supplemented with BSA / calf serum (IVP-NON-Co). Both IVP and IVD embryos were transferred non-surgically to Holstein recipients on day 7 ± 1 of the estrous cycle. Birth weight and gestation length of half-sib single calves were analyzed. No differences were observed in birth weight and gestation length between IVP-Co and IVP-NON-Co calves (31.0 kg and 31.8 kg, and 291.9 days and 291.0 days, respectively). However, the birth weight of the IVP-Co and IVP-NON-Co calves was significantly higher than that of the IVD calves ($P<0.01$). Gestation length of the IVP-Co and IVP-NON-Co calves was also significantly longer than that of the IVD calves ($P<0.01$).

KEY WORDS: birth weight, bovine embryo, gestation length, *in vitro*, large calf.

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The birth of calves following the transfer of *in vitro* produced (IVP) embryos involving *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture techniques have been reported [4, 6, 8]. Many of these studies showed increases in birth weight and gestation length [1, 7, 17] and higher incidences of dystocia, perinatal loss and anomalies in IVP calves [7].

In Japan, transfers of IVP embryos from Japanese Black cattle to Holstein recipients have been performed in order to increase meat production. However, there is limited information about birth weight and incidences of dystocia and perinatal loss among the Japanese Black calves derived from IVP embryos.

In *in vitro* culturing itself, the addition of serum to culture media and the cells used in co-culturing during *in vitro* development may be causal factors of dystocia and perinatal loss. However, the causal factors of heavier birth weight and longer gestation are unclear.

The objectives of the present study were to investigate the differences in the birth weight and gestation period of Japanese Black calves obtained from transfer of IVP embryos with (IVP-CO) or without cumulus cells (IVP-NON-CO) and embryos that had developed *in vivo* (IVD).

MATERIALS AND METHODS

Birth weight and gestation length of IVP-Co, IVP-NON-Co and IVD calves were recorded. The period of this study was from 1996 to 1999. Data concerning birth weight (measured within 24 hr after birth) and gestation length were analyzed only for single calves born.

IVM: Bovine oocytes were collected from Japanese Black cows and heifers at a local slaughterhouse and transported to the laboratory in physiological saline maintained at 33 to 35°C within 2 hr. Cumulus-oocyte complexes (COCs) were collected by aspiration of follicles 2 to 8 mm in diameter using a 10-m*l* syringe attached to a 21-gauge needle. Only oocytes with intact, unexpanded and compact cumulus cells were selected. All selected COCs were washed twice thoroughly in maturation medium consisting of Hepes-buffered TCM 199 (Gibco BRL, Grand Island, NY) supplemented with 5% heat-treated calf serum (CS; Gibco BRL). After being washed, 30 to 50 COCs were placed in the maturation medium (500 μl) in each well of a 4-well culture dish (NUNCOLON; Nunc Co., Kampstrup, Denmark) and cultured for 20 to 22 hr at 38.5°C under 5% CO_2 in air with high humidity.

IVF: One 0.5-m*l* straw of frozen semen obtained from a Japanese Black bull was used. Bovine frozen-thawed spermatozoa were washed twice with BO medium [2] supplemented with 5 mM caffeine (Caffeine Anhydrous, Wako Pure Chemical Industries, Osaka, Japan) by centrifugation at 450 g for 10 min. The sperm pellet was resuspended in the same medium in order to adjust it to a concentration of $1.0–2.0 \times 10^7$ sperm/m*l*. An equal volume of BO medium supplemented with 3 mg/m*l* bovine serum albumin (BSA; A-4378, Sigma Chemical Co., St. Louis, MO) and 10 iu/m*l* heparin (NOVO Heparin, NOVO Nordisk Pharma, Ltd., Bagsvaerd, Denmark) was added to the sperm suspension. The heparin-treated spermatozoa under mineral oil were incubated for 15 min at 38.5°C in 5% CO_2 in air. Groups of matured COCs (30–50) *in vitro* were introduced into 100-μl microdrops of sperm suspension in a 35-mm culture dish (Sumitomo Bakelite, Tokyo, Japan).

IVP-Co: After 6 hr of co-incubation, the oocytes were transferred to a development medium and cultured for 8 days with or without cumulus cells. In the co-culture groups, ten to 20 oocytes surrounded with cumulus cells

were cultured in 50-μl microdrops of Hepes-buffered TCM 199 supplemented with 5% CS and covered with mineral oil (Squibb and Sons, Princeton, NJ) at 38.5°C under 5% CO_2 in air. After 3 days of culture, uncleaved oocytes were discarded from the microdrops of the development medium and cleaved embryos were further cultured on a monolayer of cumulus cells up to Day 7 or 8.

IVP-NON-Co: 6 hr post-insemination, cumulus cells were removed from oocytes, and the cumulus-free oocytes were cultured for 3 days in a CR1aa [11] supplemented with 3 mg/m*l* BSA at 38.5°C under 5% CO_2, 7% O_2 and 88% N_2. On the third day of culture, they were transferred to a CR1aa supplemented medium with 5% CS and further cultured at 38.5°C under 5% CO_2, 7% O_2, and N_2 up to Day 8 of culture.

IVD: Superovulation was induced in Japanese Black donors (2 to 15 years old) by i. m. administration of 20 mg follicle stimulating hormone (FSH; Antrin, Denka, Kawasaki, Japan). Estrus was induced by the administration of two (24 and 12 mg) i.m. injections of prostaglandin $F_{2\alpha}$ (Pronalgon F, Takeda, Osaka, Japan) at intervals of 8 hr on the third day after the initial injection of FSH. Donors were inseminated at 12 and 24 hr after the detection of standing estrous using frozen-thawed semen, collected from the same bull as for IVP. Embryos were recovered non-surgically from 67 donors 7 days after the detection of estrus.

Transfer of embryos: Fresh and frozen embryos were transferred non-surgically to Holstein cows on Day 7 ± 1 of the estrous cycle. IVD embryos at the compact morula or blastocyct stage (Day 7 or 8) and of excellent or good quality were used for embryo transfer. IVP embryos at the blastocyst stage and of excellent quality were selected 7 or 8 days post-insemination. The IVP and IVD embryos were frozen and thawed according to the following procedure. Embryos were placed in a 1.4 M glycerol and 0.2 M sucrose mixture in PBS supplemented with 20% (V/V) CS [9, 10] and equilibrated for 20 to 30 min at room temperature (25°C). During equilibration, the embryos were individually loaded into a 0.25-m*l* straw. The cryoprotectant medium, the embryos, and 0.1 m*l* of 0.3 M sucrose in PBS supplemented with 20% CS as a dilution solution were successively loaded into the straw and separated by air bubbles, and finally the end of the straw was sealed by heating. The straws were placed directly into the precooled (–7°C) chamber of a programmable freezer (ET-1, Fujihira Kogyo, Tokyo, Japan). After 2 min, the straws were seeded at that temperature, kept at –7°C for 8 min, cooled to –23°C at 0.5°C/min and kept at –23°C for 10 min before being plunged into liquid nitrogen. The embryos were stored in LN_2 for 1 to 286 days. Thawing of the straws was accomplished by exposure to air for 5 sec followed by immersion in a 35 to 38°C water bath. After mixing, the cryoprotectant solution, 0.3 M sucrose diluent within the straws, and frozen-thawed embryos were transferred directly to the recipients.

Pregnancy was diagnosed by fetal membrane palpation through rectal inspection 60 to 70 days after estrus.

Statistical analysis: Pregnancy, abortion, perinatal and stillbirth incidence rates were analyzed by the chi-square test or Fisher's exact probability test. Data on birth weight and gestation length were analyzed using the general linear model procedure of SAS for analysis of variance and Tukey's studentized range test [12].

RESULTS

As shown in Table 1, there were differences in the pregnancy rates among the 3 types of embryos ($P<0.05$). No recipients were induced to perform parturition. However, the number of recipients that underwent labor (dystocia) for IVP-Co, IVP-NON-Co and IVD calves were 6 recipients, 3 recipients and 1 recipient, respectively. The incidence of perinatal mortality in IVP-Co calves was significantly higher than that in IVD calves (12.1% vs. 4.1%, $P<0.05$).

The birth weight and gestation lengths of IVP-Co calves were 31.0 ± 0.4 kg and 291.9 ± 1.0 days respectively, and those of IVP-NON-Co calves were 31.8 ± 0.7 kg and 291 ± 1.0 days, respectively. There were no differences in birth weights and gestation lengths of IVP-Co and IVP-NON-Co calves (Table 2). The birth weights and gestation lengths of IVP-Co and IVP-NON-Co calves were significantly higher and longer than those of IVD calves ($P<0.01$). Further, birth weights and gestation lengths in each male or female of IVP-Co and IVP-NON-Co calves were significantly higher than in each male or female of IVD calves ($P<0.05$).

Distribution of the birth weights and gestation lengths of

Table 1. Pregnancy rate and perinatal mortality for *in vitro* produced (IVP)* and *in vivo* developed (IVD) bovine embryos

Parameter	IVP-Co*	IVP-NON-Co*	IVD
No.of recipients	276	135	212
No. (%) of pregnancy	120 (43.5)[b)]	44 (32.6)[c)]	131 (61.8)[a)]
No. (%) of abortion	13 (10.8)[a)]	4 (9.1)[a)]	10 (7.6)[a)]
No.of calves (C)	107	40	121
No. (%) of perinatal death**	13 (12.1)[a)]	2 (5.0)[ab)]	5 (4.1)[b)]

a),b),c) Values in the same row with different superscripts differ significantly ($P< 0.05$). * IVP: *In vitro* produced with co-culture of cumulus cells in TCM199 supplemented with 5% calf serum (IVP-Co) or non-co-culturing without cumulus cells in CR1aa supplemented with 3 mg/m*l* BSA for the first 3 days and 5% calf serum for the next 5 days (IVP-NON-Co). ** Stillbirths and death around parturition, perinatal mortality up to 48 hr after birth because of feeble calves.

Table 2. Birth weight and gestation length following the transfer of *in vitro* produced (IVP) * and *in vivo* developed (IVD) bovine embryos

Parameter		IVP-Co*	IVP-NON-Co*	IVD
No.of calves	male	51	22	71
	female	56	18	50
	total	107	40	121
Birth weight (kg)	male	31.6 ± 6.4[c] (19–58)	31.9 ± 5.5[c] (25–52)	28.6 ± 3.1[d] (11–38)
	female	28.6 ± 5.9[e] (8–51)	29.0 ± 5.8[e] (23–60)	26.4 ± 2.8[f] (10–40)
	total	31.0 ± 6.0[a] (8–58)	31.8 ± 5.7[a] (23–60)	27.2 ± 2.9[b] (10–40)
Gestation length (day)	male	292.4 ± 5.5[a] (273-302)	290.3 ± 6.9[a] (276–299)	284.0 ± 4.6[b] (249–312)
	female	291.6 ± 4.5[a] (258–304)	292.3 ± 5.4[a] (281–303)	283.1 ± 4.7[b] (259–315)
	total	291.9 ± 4.6[a] (258–304)	291.0 ± 6.9[a] (276–303)	283.6 ± 4.6[b] (249–315)

a), b) Values (Mean ± SD) in the same row with different superscripts differ significantly (P<0.01). c),d),e),f)Values (Mean ± SD) in the same row with different superscripts differ significantly (P< 0.05). * IVP: *In vitro* produced with co-culture of cumulus cells in TCM199 supplemented with 5% calf serum (IVP-Co) or non-co-culturing without cumulus cells in CR1aa supplemented 3 mg/m*l* with BSA for the first 3 days and 5% calf serum for the next 5 days (IVP-NON-Co). ( )=range of responses.

IVP-Co, IVP-NON-Co and IVD calves are presented in Figs. 1 and 2. The percentage of calves that weighed over 36 kg at birth for IVP-Co and IVP-NON-Co was significantly higher than that for IVD (P<0.05), indicating a shift. The percentage of pregnancies lasting for over 295 days (gestation lengths) for IVP-Co and IVP-NON-Co calves was significantly higher than that for IVD (P<0.05).

DISCUSSION

In the production of Japanese Black calves by embryo transfers to Holstein recipients, transfer of IVP embryos resulted in increased birth weights of calves and longer gestation periods, which was similar to the results reported previously for other breeds [7]. Since the development of transfers of IVP embryos, the so-called "Large Offspring Syndrome" has been reported [17]. Of course, not all offspring are affected [7]. In the present study, we confirmed the increase in birth weights of calves produced by transfer of IVP embryos and the high incidence of stillbirth and perinatal mortality up to 48 hr postpaturation in this group. These results may suggest the existence of "Large Calf Syndrome" in Japanese Black calves produced by transfer of IVP embryos. Further investigation into the relationship between increased birth weights and the high incidence of stillbirth and perinatal mortality is needed.

It is not clear what factors cause the higher birth weight and longer gestation period. Techniques that can lead to a predisposition to fetal oversize include embryo culture [14, 15], embryo-somatic cell co-culture [3] and asynchronous embryo transfer [16], but the causal factors have not been identified. It is thought that the nature of these abnormalities is associated with the biological differences between *in vivo*- and *in vitro*-produced embryos [18]. Farin and Farin [3] have reported that at 7 months of gestation, fetuses from IVP embryos produced by co-culture with oviductal epithelial cells in TCM 199 supplemented with 10% estrous cow serum were heavier than fetuses from IVD embryos. We found no significant differences of birth weight and gestation length between IVP-Co and IVP-NON-Co calves, though the birth weight and gestation length of calves born after transfer of both IVP embryos was heavier and longer than those born after transfer of IVD embryos in Japanese Black cattle. It is well known that co-culture cells secrete embryotrophic growth factors [13]. Also, serum may contain some components such as growth factors or fibronectin [19]. Recently, Van Wagtendonk-de Leeuw *et al.* [14] reported that calves born after transfer of IVP embryos produced in SOF medium supplemented with BSA had significantly lower birth weights than those of IVP-Co embryos produced by co-culture with BRL cells in serum containing medium. It could be inferred that the serum supplement in the *in vitro* culture media is a factor contributing to the difference observed in birth weight, however, further investigation is needed to verify this.

In this study, the incidences of stillbirth and perinatal death in IVP calves were significantly higher than those in IVD calves. Garry *et al.* [5], and Kruip and den Daas [7] reported that transfer of IVP embryos caused problems in perinatal mortality and calf viability. The causal factor for these problems is believed to be the conditions of *in vitro* maturation, *in vitro* fertilization and *in vitro* culturing. Furthermore, with regard to the higher incidence of stillbirth, there is a need for a detailed investigation of the individual immunological functions of IVP calves [20].

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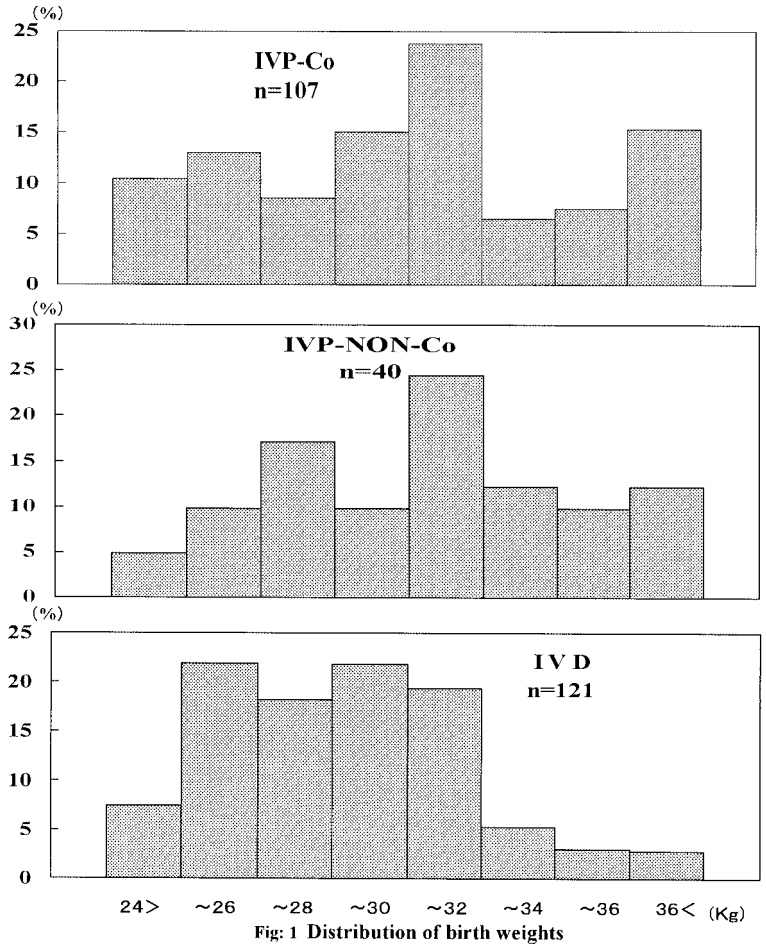

Fig. 1. Birth weights of Japanese Black calves originating from either IVP (*in vitro* produced) with co-culture of cumulus cells in TCM199 supplemented with 5% calf serum (IVP-Co), non-co-culturing without cumulus cells in CR1aa supplement with 3 mg/m*l* BSA for the first 3 days and 5% calf serum for the next 5 days (IVP-NON-Co), and IVD (*in vivo* developed).

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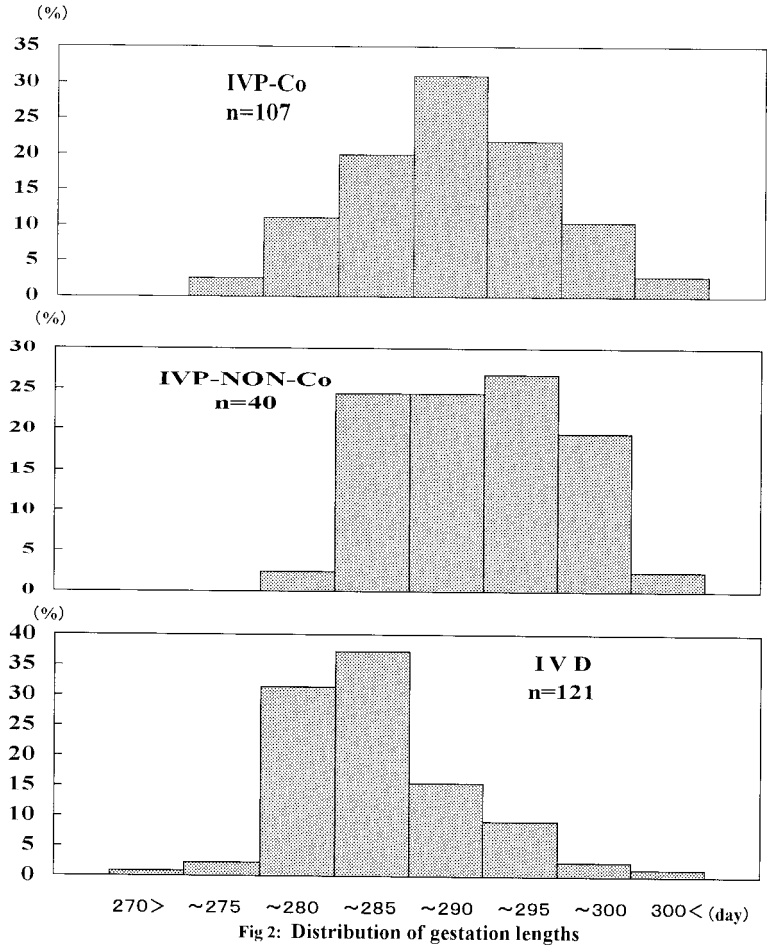

Fig. 2. Gestation lengths of Japanese Black calves originating from either IVP (*in vitro* produced) with co-culture of cumulus cells in TCM199 supplemented with 5% calf serum (IVP-Co), non-co-culturing without cumulus cells in CR1aa supplement with 3 mg/m*l* BSA for the first 3 days and 5% calf serum for the next 5 days (IVP-NON-Co), and IVD (*in vivo* developed).

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