FULL PAPER *Theriogenology*

Improvement of Superovulatory Response and Pregnancy Rate after Transfer of Embryos Recovered from Japanese Black Cows Fed Rumen Bypass Polyunsaturated Fatty Acids

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ABSTRACT. Feeding rumen bypass polyunsaturated fatty acids (PUFA) affects to Japanese Black cows affects their reproduction, though its influence on superovulatory response in donor cows and conception in recipient cattle has not been well studied. Here, we investigated the effects of feeding PUFA to Japanese Black cows on blood biochemistry, the numbers of ova and embryos or transferable embryos and pregnancy rate following embryo transfer (ET) to recipient Holstein heifers. PUFA (40% linoleic acid) was fed at 300 g/day in the experimental group from the last day of estrus until the day of artificial insemination for superovulatory treatment. Blood was collected on the first day of follicle-stimulating hormone administration. Total cholesterol level was significantly higher in the 15- to 19-day feeding group (117.4 mg/d*l*) than in the control group (95.0 mg/d*l*). The numbers of ova and embryos or transferable embryos were significantly higher in the 15- to 19-day feeding group than in the control group. The numbers of transferable embryos in the 15- to 19-day feeding group were significantly higher than in the 10- to 14-day feeding group. The pregnancy rate at day 60 was significantly higher in the experimental group (66.7 and 57.1%) than in the control group (51.1 and 44.0%) after transfer of fresh and frozen-thawed embryos, respectively. In conclusion, the numbers of ova and embryos or transferable embryos after superovulatory treatment increased, and the pregnancy rate after ET was higher in Japanese Black cows fed PUFA than in the control group.

KEY WORDS: embryo transfer, Japanese Black cow, linoleic acid, polyunsaturated fatty acid, superovulatory response.

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Induction of multiple ovulations and embryo transfer (ET) in cattle are important techniques that enable production of increased numbers of excellent calves from oocytes produced by superior dams using the semen of sires having excellent genotypes. However, oocyte and embryo quality after superovulatory treatment is markedly affected by the nutritional status of the donor cows, feeding management techniques and roughage quality.

The postpartum energy balance affects the reproductive performance of donor cows. A negative energy balance causes reproductive problems, such as reduction of LH pulse frequency, interference with follicle maturation and delayed ovulation [5]. Endocrine changes associated with energy balance and lactation [5, 27] and localized effects of metabolites in the follicular fluid [14] may compromise oocyte and embryo quality and developmental competence in dairy cows. However, the exact mechanisms of these effects remain unclear, and it is not fully known how the nutritional status of the donor Japanese Black cow influences oocyte and embryo quality and pregnancy rate after ET.

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Fat supplementation influences reproduction by affecting the size of the dominant follicle, shortening the interval to first postpartum ovulation in cattle, increasing the progesterone concentration during the luteal phase, modulating uterine prostaglandin synthesis and improving oocyte and embryo quality and developmental competence [20]. Some of these effects are altered by the type of fatty acid fed. Rumen bypass polyunsaturated fatty acids (PUFA) of the n-6 (linoleic acid, C18:2) and n-3 [α-linolenic acid, C18:3; eicosapentaenoic acid (EPA), C20:5; docosahexaenoic acid (DHA), C22:6] families have remarkable effects on reproduction in cattle [20], but it is not known whether these effects are mediated only by these fatty acids or by other potential intermediates produced during rumen biohydrogenation.

Rumen bypass fat supplementation increased the conception rate to first service, but did not significantly affect pregnancy rates at the end of the breeding season in dairy cows [16]. Supplementation with a rich source of n-3 fatty acids decreased the quality of embryos from donor lactating dairy cows compared with supplementation with calcium salts of palm oil, but it had no effect on the subsequent pregnancy rate of recipient heifers receiving frozen grade-1 embryos of the donor dairy cows [18]. A sunflower seed diet rich in linoleic acid had no effect on the numbers of ova and embryos or the number of transferable embryos in dairy cows [25]. Supplemental rumen bypass fat fed to primiparous beef heifers did not improve their fertility in the breeding season [8].

As mentioned above, there has not been a great deal of

research on the effects of fat supplementation on fertility. Feeding unsaturated fatty acids (UFA) improved embryo quality and development in Holstein dairy cows [7, 25]. However, there are no reports on multiple ovulation and ET after UFA administration in Japanese Black cows. Here, we investigated the effects of feeding PUFA to Japanese Black cows on blood biochemistry, the total numbers of ova and embryos and the number of transferable embryos recovered after superovulatory treatment and pregnancy rates following ET.

MATERIALS AND METHODS

Cows and experimental diets: Postpartum nonlactating and lactating Japanese Black cows at Koiwai Farm in Iwate Prefecture were used in this study. The animals were fed roughage consisting of orchard grass hay and hay silage *ad libitum*, a concentrate specially formulated for Japanese Black cows (1.5 kg/day) and wheat bran (1.5 kg/day). The Japanese Black cows were randomly allocated to an experimental group (n=50) and a control group (n=50). PUFA product (Tomalinole; Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) was fed at 300 g/day (180 g Ca salts of fatty acid including 40% linoleic acid and 75 mg β-carotene) in the experimental group from the last day of estrus until the day of artificial insemination for superovulatory treatment (for 15.2 ± 2.3 days; min 10 days, max 19 days). In addition, the PUFA feeding days were classified into 2 groups, 10 to 14 days and 15 to 19 days, and it was compared with the feeding days of PUFA about relationship of the total numbers of ova and embryos and the number of transferable embryos. Tomalinole comprised 6% crude protein, 52% crude fat, 4.3% crude fiber, 10.5% crude ash, 5.2% digestible crude protein (DCP), 132% total digestible nutrients (TDN), 6.1% Ca and 0.1% P. The diet of the control group had the same nutritional composition as the experimental diet, except that it did not contain PUFA, and feeding management was the same in both groups.

Blood biochemistry tests: In the experimental and control groups, blood was collected from the tail vein or artery on the first day of follicle-stimulating hormone (FSH) administration, and serum was separated by centrifugation. Blood biochemical tests were carried out with a SPOTCHEM EZ SP-4430 autoanalyzer (Arkray Co., Ltd., Kyoto, Japan) after embryo recovery. Serum concentrations of glucose (Glu), total cholesterol (T-Cho), blood urea nitrogen (BUN), aspartate amino transferase (AST), total protein (T-Pro) and albumin (Alb) were determined. Serum β-carotene concentrations were analyzed as described previously [21] after one-step denaturation and extraction into organic solvent using the iEx™ assay system and a portable photometer (iCheck™; BioAnalyt GmbH, Teltow Germany). The analysis consisted of three simple steps: injection, reaction and measurement within a few min. Extraction efficiency was>95%. The intra- and interassay coefficients of variation averaged 7.4 and 8.3%, respectively.

Superovulatory treatment, embryo recovery and freezing: The superovulatory treatment consisted of twice daily intramuscular injections of decreasing doses of FSH (Antorin R•10; Kyoritsu Seiyaku Corporation, Tokyo, Japan) for 3 days with an initial dose of 5 AU 5 to 13 days after estrus and a total dose of 24 AU [10]. Donors received cloprostenol 0.5 mg (a $PGF_{2\alpha}$ analog, Resipron-C; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) 48 hr after the initial injection of FSH. Donors were inseminated 12 hr after estrus for embryo recovery. Superovulated donors were inseminated artificially with frozen semen once following onset of estrus. Occasionally, donors were inseminated, if they showed signs, such as vaginal mucus discharge and increased physical activity, even though standing estrus was not observed. Seven days after estrus, embryos were collected nonsurgically in commercial flushing medium (Embryotech; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan). The flushing fluid recovered from the uterus was passed through an embryo collection filter (Embryo Collector; Fujihira Industry Co., Ltd., Tokyo, Japan) and searched for ova and embryos under a stereoscopic microscope. Embryos were classified according to the Manual of the International Embryo Transfer Society [24]. Coded 1 (excellent/good) or coded 2 (fair) embryos at developmental stages 4 (morula) to 7 (expanded blastocyst) were considered transferable embryos. The numbers of unfertilized ova, untransferable embryos and transferable embryos were examined. The cryoprotectant for the direct transfer of frozen-thawed embryos was 1.5 M ethylene glycol (EG) and 0.1 M sucrose in Dulbecco's phosphate-buffered saline (+) (D-PBS; Gibco, Life Technologies, Carlsbad, CA, U.S.A.) supplemented with 20% heat-inactivated calf serum from calves born on Koiwai Farm. The embryos were equilibrated in 1.5 M EG containing 0.1 M sucrose for 5 to 15 min at room temperature. During equilibration, the embryos were loaded individually into 0.25 m*l* straws (IMV Technologies, L'Aigle, France). The straws were placed directly into the precooled chamber of a programmable freezer (ET-U3; Fujiya Yano Science, Sapporo, Japan) at −7°C. The straws were seeded automatically, preserved at −7°C for 10 min and cooled to −30°C at 0.3°C/min before being plunged into liquid nitrogen. The straws were thawed by allowing them to stand in air for 5 to 10 sec, followed by immersion in a 30°C water bath until all ice melted.

Embryo transfer and pregnancy diagnosis: Most recipients were used for ET after observation of standing estrus, and the remaining recipients were used for synchronization of estrus with 0.5 mg of the $PGF_{2\alpha}$ analog. ETs were performed on days 6 to 8 (day 0 =onset of estrus). The recipients were Holstein heifers with a corpus luteum (CL) having a palpable diameter of more than 12 mm and a firm or moderately firm consistency by rectal palpation. Transferable embryos were selected at random, and stages in the development of the transferable embryos were not inclined among the three groups. Fresh embryos were transferred into the uterine horn ipsilateral to the ovary bearing the CL. Frozen-thawed embryos without diluting EG in the cryoprotectants were transferred in the same way. One embryo per recipient was nonsurgically transferred with an ET syringe having a 3-mm diameter sheath and a shallow chamber (IMV Technologies). Pregnancy following ET was diagnosed by rectal

Table 1. Serum biochemical findings and β-carotene concentrations in Japanese Black cows after feeding bypass polyunsaturated fatty acids (PUFA)

Parameters	Control group (n=50)	Experimental group 10 to 14 days feeding (n=15)	Experimental group 15 to 19 days feeding (n=35)
Glu (mg/d*l*)	64.1 ± 8.2	66.9 ± 5.9	62.0 ± 9.1
T-Cho (mg/d*l*)	95.0 ± 25.1[a)]	112.2 ± 25.4[a,b)]	117.4 ± 27.3[b)]
BUN (mg/d*l*)	14.2 ± 3.5	13.3 ± 3.7	14.1 ± 3.4
AST (IU/*l*)	66.3 ± 31.2	60.1 ± 15.5	59.4 ± 14.1
T-Pro (g/d*l*)	6.8 ± 0.6	6.6 ± 0.6	6.8 ± 0.6
Alb (g/dl)	3.5 ± 0.2	3.4 ± 0.3	3.5 ± 0.3
β-carotene (mg/*l*)	2.0 ± 1.1	2.3 ± 1.0	2.2 ± 0.7

Blood samples were collected from donors on the first day of superovulatory treatment. Values are means ± SD of donors. a, b) Values with different superscripts within the same row indicate significant differences ($P<0.001$).

Table 2. Relationship between the feeding days of bypass PUFA supplement and the numbers of ova and embryos or transferable embryos recovered from Japanese Black cows

Group	No. of donor cows	No. of ova and embryos per cow	No. of transferable embryos per cow*
Control	50	10.0 ± 7.6 [a)]	4.5 ± 3.9 [a)]
Experiment			
10 to 14 days feeding	15	10.1 ± 9.0[a,b)]	4.1 ± 3.9[a)]
15 to 19 days feeding	35	14.9 ± 8.9[b)]	7.8 ± 6.0[b)]

Embryos were collected 7 days after estrus. * Morula to expanded blastocyst with code 1 or 2. Values are means ± SD of donors. a, b) Values with different superscripts within the same column are significantly different ($P<0.05$).

palpation on day 60 after estrus.

Statistics analysis: Data are reported as means ± standard deviation. The values followed by multiple pair-wise comparisons were assigned for one-way analysis of variance (ANOVA) with Tukey's test. The pregnancy rate following ET was defined as the number of pregnant cows divided by the number of treated cows. Differences in pregnancy rate between two groups were examined with the Chi-square test. Significance was declared at $P<0.05$. All data were analyzed using SPSS (SPSS version 17.0J; SPSS Japan Inc., Tokyo, Japan).

RESULTS

Blood biochemistry and β-carotene concentration in donors: Blood biochemical traits and β-carotene concentrations in the donors are shown in Table 1. The level of T-Cho was higher ($P<0.001$) in the 15- to 19-day feeding group (117.4 ± 27.3 mg/d*l*) than in the control group (95.0 ± 25.1 mg/d*l*). Other biochemical values did not differ significantly among the three groups.

Embryo recovery after feeding bypass polyunsaturated fatty acids: Results of embryo recovery from Japanese Black cows after feeding PUFA are shown in Table 2. Transferable embryos were recovered from 90.0% (45/50) of the experimental cows and 84.0% (42/50) of the control cows. The relationship between feeding days of bypass PUFA supplement and the numbers of ova and embryos is shown in Table 2. The total numbers of ova and embryos per cow in the 15- to 19-day feeding group (14.9 ± 8.9) were significantly higher than those in the control group (10.0 ± 7.6). The number of transferable embryos per cow was significantly higher in the 15- to 19-day feeding group (7.8 ± 6.0) than in the control group (4.5 ± 3.9). The numbers of ova and embryos in the group fed the bypass PUFA supplement for 15 to 19 days were significantly higher than in the control group, but were not different from those in the 10- to 14-day feeding group. The number of transferable embryos in the group fed the PUFA supplement for 15 to 19 days was significantly higher than those in the 10- to-14 day feeding group and control group.

Pregnancy rate following embryo transfer: Pregnancy rates are shown in Table 3. The pregnancy rate in the experimental group (66.7%) was significantly higher than that in the control group (51.1%) after transfer of fresh embryos. The pregnancy rate in the experimental group (57.1%) was significantly higher than that in the control group (44.0%) after transfer of frozen-thawed embryos.

DISCUSSION

It was previously suggested that the PUFA content of a diet can influence both ovarian and uterine function in cows [19]. The diameter of the first dominant follicle, the insulin-

Table 3. Pregnancy rate of Holstein heifers following transfer of embryos recovered from Japanese Black cows with bypass PUFA feeding

Group of donor cows	Pregnancy rate after transfer of each type of embryos	
	Fresh embryos	Frozen-thawed embryos
Control	51.1% (46/90) [a)]	44.0% (59/134) [a)]
Experiment	66.7% (46/69) [b)]	57.1% (72/126) [b)]

Pregnancy rate: no. of pregnant recipients / no. of recipients. a, b) Values with different superscripts within the same column are significantly different ($P<0.05$).

like growth factor I (IGF-I) concentration at estrus and cholesterol concentrations were all higher in cows fed a diet supplemented with linoleic acid (18:2, n-6) than in cows that did not receive this supplement [19]. Dietary UFA increased P450 aromatase mRNA expression in granulosa cells and elevated steroid hormones, such as estradiol, in preovulatory follicles, which may be beneficial to subsequent ovarian function [29]. In the present study, the increase in the total numbers of ova and embryos in Japanese Black cows indicates an increased number of ovulated follicles. Increased serum T-Cho concentrations may stimulate ovarian production of steroids, such as estradiol during the follicular phase, because cholesterol is a precursor of steroid sex hormones. An elevated estradiol level may promote the development of medium-sized follicles in superovulatory treatment.

A high level of inert fat in the rumen improved blastocyst production from mature and cleaved oocytes, and blastocysts from a high-fat group had a significantly higher total number of inner cell mass and trophectoderm cells than those from a low-fat group [9]. Fertilization rate, number of accessory spermatozoa and proportion of grade 1 and 2 embryos were all improved in nonsuperovulated dairy cows receiving the mixture of linoleic acid and trans fatty acids (C18:1) from 25 days prepartum to 80 days in milk as compared with cows receiving calcium salt of palm oil distillate [6]. The number of transferable embryos was considerably influenced for the feeding period, because the number of transferable embryos was significantly higher in the 15- to 19-day feeding group than in the control group and 10- to 14-day feeding group. The PUFA supplement was fed for 10 to 19 days from the last estrus day until the day of artificial insemination for superovulatory treatment. In the future, it is necessary to study whether the numbers of transferable embryos increase, if the feeding period of PUFA is extended to more than 20 days.

The T-Cho level was significantly higher in the 15- to 19-day feeding group than in the control group. Other biochemical variables did not differ significantly among the three groups. In a study in which the blood cholesterol level of donor cattle was high, the proportion of good-quality embryos was also high [13]. In the present study, the significantly higher total numbers of ova and embryos and number of transferable embryos in the 15- to 19-day feeding group were associated with a higher level of T-Cho. All groups received *ad libitum* feeding of hay and hay silage roughage and had normal blood levels of biochemical variables. On the other hand, short- or long-term overfeeding with a high intake has negative effects on embryo yield and quality following superovulation in nonlactating cows [22] and heifers [17, 28]. The increases in the total numbers of ova and embryos and the number of transferable embryos may have been related more to the linoleic acid included in PUFA than to the additional energy resulting from PUFA intake.

Iwanska *et al.* [11] reported improvements in fertility, such as in the number of inseminations per conception and the conception rate, in cows after β-carotene supplementation. On the other hand, β-carotene supplementation did not improve fertility in Holstein cows [2, 4, 26]. Kawashima *et al*. [12] supplied cows with 2,000 mg of β-carotene daily by oral administration during the close-up dry period. Supplying β-carotene may support the onset of luteal activity during early lactation in dairy cows. The influence of β-carotene on embryo recovery and embryo quality was not clear in our study, because the β-carotene levels in the experimental groups were not significantly higher than in the control group for the short period (about 15 days) or low dosage of the supplied β-carotene.

Changes in chain length and the degree of unsaturation and position of the double bonds in the acyl chain of fatty acids can have marked effects on their function and may affect reproduction in cows [23], although the potential implications for developmental quality of *in-vitro* embryos are less clear [1, 3]. The total cell number of embryos recovered and embryonic development were improved in Holstein cows fed UFAs compared with those fed saturated fatty acids [25]. Dietary n-3 fatty acids influenced follicular status and increased the cleavage rate of oocytes *in vitro* in cows [30]. Treatment of bovine cumulus-oocyte complexes with α-linolenic acid of n-3 fatty acid during oocyte maturation affected the molecular mechanisms controlling oocyte nuclear maturation, leading to an increased rate of oocyte maturation and improved subsequent early embryo development *in vitro* [15]. These effects are mediated both directly through the mitogen-activated protein kinase pathway and indirectly through PGE2 synthesis [15]. In the case of Tomalinole, which is rich in linoleic acid, an n-6 fatty acid, feeding the PUFA supplement has a positive effect on the quality of the embryo, because increment of the linoleate blood level may have an influence on oocyte maturation and embryo development. We think that the conception rate of recipients after transfer of embryos from Japanese Black donor cows fed PUFA was improved by the better developmental quality of the embryos. The effect of PUFA can be important for improvement of embryo quality and viability, especially after cryopreservation.

In conclusion, the total numbers of ova and embryos and the number of transferable embryos after superovulatory treatment were increased by feeding PUFA for 15 to 19 days, and the pregnancy rate after ET was significantly higher in Japanese Black cows fed PUFA than in the control group. The number and quality of ova and embryos after superovulatory treatment ware affected by the feeding period of a bypass PUFA, and long-term feeding was more effective than a short-term feeding. Supplementation with PUFA thus improves superovulatory response and leads to the production of high-quality, viable embryos in Japanese Black cows.

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