Developmental Competence of Oocytes Collected from Individual Ovaries of Slaughtered Korean Native Cattle with Grade of Meat Quality and Meat Yield

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ABSTRACT

We separately cultured follicular oocytes collected from individual ovaries of slaughtered Korean native cows and examined both the embryonic development rate and pregnancy rate after embryo transplantation according to the meat yield and quality grades of the source beef carcass. Oocytes from meat yield grade B cows exhibited a higher fertilization rate and embryonic developmental rate to the eight-cell stage than oocytes from grade A or C animals ($p<0.05$), but there was no significant difference in rate of development to the blastocyst stage among meat yield grades A, B and C. The oocyte cleavage rate and development rate to the eight-cell stage from meat quality grade 3 cattle was higher than grades 1++, 1+, 1 and 2 ($p<0.05$). Embryos derived from grade 3 animals displayed a development rate to the blastocyst stage of 19.4%, which was also higher than all other meat quality grades ($p<0.05$). Transplantation of *in vitro*-cultured oocytes from meat yield grade A ovaries led to a higher pregnancy rate (64.2%) than *in vitro*-cultured oocytes from meat yield grade B ovaries (56.5%), but there was no significant difference between the two groups in pregnancy or abortion rates.

In conclusion, embryonic development rate and pregnancy rate has a close relation to meat quality grades of the source beef carcass, this results is to give information for the Korean native cows improvement of breed.

(Key words : bovine embryo, meat quality, meat yield, carcass grade, individual ovary collection)

INTRODUCTION

In embryonic transfer (ET), a number of embryos are recovered from cows with high genetic merit and transplanted into identical or other species, resulting in the production of a calf. Although this method enhances reproductive efficiency and allows proliferation of individuals with excellent genetic character, it has been sparingly used due to problems such as high costs and inefficiency, among others. Since Brackett *et al.* (1982) reported the successful production of a calf from immature follicle oocytes recovered from the ovary of a slaughtered cow, the study of reproductive techniques involving *in vitro*-produced embryos has advanced, making it possible for 20~30% of *in vitro*-fertilized oocytes to develop to the blastocyst stage, when they can be transferred. The primary goal for embryo transfer is pregnancy. Although pregnancy rates may vary slightly depending on the embryo grade, *in vitro*-produced embryos have a pregnancy rate of 30±10% (Peterson and Lee, 2003), which is lower than artificial insemination (AI) (79%; Xu *et al.*, 1995) and *in vivo*-produced embryos (64%; Schmidt *et al.*, 1996). Reproducibility is very low due to recipient selection, operator experience, and proper nutritional management. Moreover, the abortion rate of recipients with *in vitro*-produced embryos is 9~47% (Schmidt *et al.*, 1996), much higher than that of AI and *in vivo*-produced embryos (4~8%; Hasler *et al.*, 1987). Calf body weight, gestation period, hard labor, and

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deformity rate are also higher with *in vitro*-derived embryos (Kruip *et al.*, 1997).

In current practice, the ovaries of slaughtered cows are typically mixed for *in vitro* embryo production (Schmidt *et al.*, 1996; Numabe *et al.*, 2000; Peterson and Lee, 2003) or oocytes are aspirated by sonography from cows that are normal, pregnant (Meintjes *et al.*, 1995; Reinders *et al.*, 1996), propagation obstacles (Pieterse *et al.*, 1992), or immature (Tervit, 1996; Fry *et al.*, 1998). During aspiration, *in vivo* follicular oocytes can be periodically collected by sonography, and it is possible to know the pedigree and characteristics of the maternal line, making it a very effective method for livestock improvement. However, the harvest of follicular oocytes by sonography is used less often than the aspiration of follicular oocytes from slaughtered cows due to the recovery rate, high variation in grade of the recovered follicular oocytes, expensive equipment, and technical difficulties. In contrast, the use of *in vitro*-produced embryos from slaughtered cow ovaries has the potential for easy mass production through the mobilization of a discarded genetic resource. At the same time, a system that uses the recovered, combined, and randomized follicular oocytes from various ovaries cannot take into account maternal genetic characteristics such as meat quality or meat yield, making it less advantageous for livestock improvement. These shortcomings can be overcome, however, by individually culturing recovered follicular oocytes from the ovary of a single slaughtered cow with verified meat quality grade and meat yield grade. Using this method, the production and transfer of embryos with excellent hereditary factors could accelerate the improvement of native Korean cows. We individually cultured recovered follicular oocytes from known cows and examined rates of embryo development and pregnancy after embryo transfer according to the genetic characteristics of the female donor.

MATERIALS AND METHODS

1. Individual Ovary Collection

Bovine ovaries were collected at an abattoir, and blood and other foreign materials were removed from the outer layer of ovaries. The harvested ovaries were placed in a labeled polyamide pack and transported within 2~3 h to the laboratory in a vacuum bottle containing saline and antibiotics (Penicillin G; P3032, Sigma, USA). The next day, the meat quality grade and meat yield grade (as decided by Youngnam LPC Co., Changryeong, Korea) of the slaughtered cows were recorded. Meat quality was graded as 1++, 1+, 1, 2 and 3 by its marbling score, meat color, fat color, tenderness, and maturity. Meat yield grade (A, B and C) represented the percent meat yield from the slaughtered cows according to carcass weight, back fat thickness, and eye muscle area.

Care and treatment of all animals in this study was approved by the Ethical Committee of medical center of Daegu CHA general hospital.

2. Culture Media

All follicular oocytes were collected and washed in DPBS (Gibco BRL, Grand Island, NY) supplemented with 0.3% (w/v) bovine serum albumin (BSA; A6003, Sigma). TCM-199 supplemented with 10% FBS, 1 μg/ml follicle stimulation hormone (FSH, F8174, Sigma, USA) and 1 μg/ml 17β-estradiol (E2758, Sigma, USA) was used as a culture media for *in vitro* maturation (IVM) of immature oocytes. The culture media Sperm TALP (SP-TALP; Parrish *et al.*, 1988) was used for sperm washing and Fert-TALP (Bavister *et al.*, 1977) was used for *in vitro* fertilization (IVF). CR-1 supplemented with 0.3% (w/v) BSA culture media was used for *in vitro* culture (IVC) of early-stage embryos (2~8 cell stage), and CR-1 supplemented with 10% (v/v) FBS was used in late-stage embryo (8 cell~blastocyst stage) culture. Solutions expressed as percents were prepared as volume-to-volume (v/v) dilutions. All media used for IVM, IVF, and IVC were incubated at 38.5℃ and 5% CO_2 with maximum humidity for 4 h before use. We attempted to identify the most effective medium for individual embryo culture by evaluating a variety of media. The IVM medium for immature oocytes (IVMD 101), IVF medium (IVF 100), and IVC medium (IVD 101) were used as complete serum-free culture media (all from the Research Institute for Functional Peptides, Japan; Yamashita *et al.*, 1996). For sperm washing and IVF, IVF 100 media was used with components partially modified from the BO culture medium (Brackett and Oliphant, 1975), including 25 mM sodium pyruvate, 0.5 mM cysteine, 5 mg/mL BSA, 5 mM caffeine (Wako Pure Chemical Industries, Ltd., Oska, Japan), and 7.5 mg/ml heparin.

All culture media were prepared 2 weeks before use and filtered with 0.22 μm membrane filters (Gelman Science, Ann Arbor, MI). The media were stored at 4℃ and equilibrated for 4~5 h in an incubator with 5% CO_2 before use.

3. Collection of Follicular Oocytes

The ovaries of Korean native cattle (KNC) were individually

obtained at an abattoir and separately washed 2~3 times with saline solution. Blood and other foreign materials were removed from the surface of the ovaries, and the follicular oocytes were collected by aspiration from 2~6 mm follicles with an 18-gauge needle attached to a 10 ml disposal syringe. Oocytes were washed 2~3 times in DPBS supplemented with 0.3% (w/v) BSA and the upper layer of solution was removed. The follicular oocytes were collected under a stereoscopic microscope with a Pasteur pipette and divided individually into labeled culture drops on a 35 mm Petri dish.

4. *In Vitro* Maturation of Follicular Oocytes

Individually collected follicular oocytes were washed 1~2 times in IVM media and placed into 50 μl of IVM media with 1~40 follicular oocytes per culture drop and incubated for 22~24 h with Multi-gas (5% CO_2, 90% N_2 and 5% O_2) at 39℃ and 95% humidity. Subsequently, the expanded cumulus cells of the follicular oocytes were removed for IVF.

5. Sperm Preparation and *In Vitro* Fertilization

Frozen semen (−196℃ LN_2) straws from KNCs were thawed by warming in air for 10 sec and exposure to 39℃ water for 20 sec. Both ends of each straw were cut with sterilized scissors and the semen was placed in a 15 ml centrifuge tube (Falcon, 2097) containing 2 ml of 45% and 2 ml of 90% Percoll solution in SP-TALP medium. Samples were centrifuged at 700×g for 20 min, and the supernatant was discarded. Pellets were collected and washed twice in SP-TALP medium at 350×g for 5 min, then resuspended to a final concentration of 1×10^6 spermatozoa/ml. After the IVM process, in which the follicular oocytes were cultured in 50 μl drops of Fert-TALP media for 22 h in a 60 mm Petri dish covered with mineral oil, the oocytes were washed 1~2 times and incubated with sperm for 24 h at 39℃ with 5% CO_2 in an incubator for IVF. IVF 100 was used as a serum-free culture medium for both IVM and IVF.

6. *In Vitro* Culture of Embryos

After IVF for 24 h, the cumulus cells of individual follicular oocytes were stripped off by pipetting with a 200 μl micropipette, leaving the third layer of cells. The fertilized oocytes were collected based on normal morphology with/without the presence of two pronuclei (PN) under a stereoscopic microscope. Embryos collected from same KNC were washed 2~3 times in 50 μl drops of CR-1 medium supplemented with BSA that were prepared on a 60 mm Petri dish covered with mineral oil. The embryos were then divided into groups of 1~40 embryos from a single cow per drop and cultured in a 60 mm dish (Falcon, 3002) containing 30 μl drops of culture medium. After 48 h of culture, the culture medium was changed to CR-1 medium supplemented with 10% (v/v) FBS, which was then refreshed every 48 h. The cultures were continuously carried out for 7~8 days, and embryonic development was verified to the blastocyst stage.

7. Embryo Transfer (ET)

Blastocyst stage or expended blastocyst stage embryos were selected after *in vitro* culture for 7~8 days. This study was conducted on 58 farms located in Kyongbuk bovine, Korea.

The recipients for embryo transfer included healthy F-1 heifers (KNC × Holstein, n=229), and milking cows more than 14 months old. Heifers were selected for ET if they had experienced at least three normal estrous cycles. A rectal examination was carried out 6~7 days after the onset of estrus (day 0) to identify the state of the uterus and ovary and the position of a corpus luteum (left or right). Recipients with a corpus luteum of normal size (15~25 mm) and morphology were injected intramuscularly twice every 11 days with 25 ml of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$: Lutalyse, Upjohn Co.) to induce sexual excitement. Once sexual excitement was induced, the recipients were observed once in three times and more than 30 min in each time to allow the selection of individuals with standing estrus (estrus Day 0). Embryos were transferred non-surgically by an endocervical approach to the recipient 7~8 days after the onset of estrus.

To investigate the developmental capacity and fertility rate of *in vitro* fertilization, recipients were classified into F1- and milk cow-transferred embryos. Pregnancy was diagnosed by rectal palpation or sonography on day 50~60 after embryo transfer.

8. Statistical Analysis

Results were analyzed using the χ^2-test with SPSS software. A P value <0.05 was considered statistically significant.

RESULTS

Effect of number of follicular oocytes aspirated per ovary on *in vitro* development. *In vitro* embryo developmental rates according to the number of follicular oocytes aspirated from individual ovaries is presented in Table 1. Groups of 1~5 or

5~10 follicular oocytes had developmental rates (to the two-cell stage) of 73.2 and 70.2%, respectively, which were significantly higher than groups with more than 10 follicular oocytes. Smaller groups also had higher rates of development to the eight-cell stage. The group with 5~10 follicular oocytes displayed a higher blastocyst rate than any other group.

Effect of serum-free media on *in vitro* oocyte development. For controls, the aspirated follicular oocytes were individually cultured in drops with classified medium, medium with serum, or serum-free media for IVM and IVF or a combination of serum media for IVC. The resulting embryo developmental rates according to the type of *in vitro* culture media for follicular oocytes is shown in Table 2. Serum-free media yielded a developmental rate to the two-cell stage of 56.9%, and supported a significantly higher cleavage rate than media with serum. Although serum or combination serum media produced higher developmental rates to the eight-cell and blastocyst stages than serum free-media, the difference did not reach statistical significance. The developmental rate of grade 1 and 2 blastocysts was 55.4% in serum-free media, which was significantly higher than oocytes grown in combination serum or serum media (42.0 and 34.7%, respectively).

Effect of high meat yield grade on *in vitro* oocyte development. We examined *in vitro* oocyte development from recovered follicular oocytes according to the meat yield grade of the source animal (Table 3). In cattle of meat yield grade A, an average of 18.4 follicular oocytes was collected from the ovaries of each individual cow, compared to 17.0 and 18.5 follicular oocytes from grades B and C cows, respectively. There was no significant difference in oocyte collection based on meat yield grade. The developmental rates to two- and eight-cell stages were higher in oocytes from grade B animals (58.7 and 45.8%, respectively) than grade A and C animals ($p<0.05$), although there was no significant difference in the blastocyst developmental rate based on meat yield grade (15.3~ 15.6%).

Effect of meat quality grade on *in vitro* oocyte development. We also evaluated *in vitro* oocyte development of recovered follicular oocytes from ovaries of slaughtered KNC based on the meat quality grade of the animals (Table 4). Animals with the best meat quality grade (1++) also yielded the greatest number of follicular oocytes (18.9), but the difference

Table 1. Effect of the number of follicular oocytes aspirated per individual ovary on the development of *in vitro* oocytes

No. of oocytes	No. of ovaries	No. of aspirated oocytes (per ovary)	No. (%) of embryos development to		
			≥2-cell	8-cell	Blastocyst
1~5	209	758 (3.6)	555 (73.2)[a]	427 (56.3)[a]	127 (16.8)[ab]
5~10	369	3,001 (8.1)	2,107 (70.2)[a]	1,623 (54.1)[a]	548 (18.3)[a]
10~15	313	4,065 (13.0)	2,624 (64.6)[b]	2,031 (50.0)[b]	709 (17.4)[a]
15~30	513	11,236 (21.9)	6,565 (58.4)[c]	5,051 (45.0)[c]	1,699 (15.1)[b]
30~	235	11,032 (47.0)	5,136 (46.6)[d]	3,873 (35.1)[d]	1,481 (13.4)[c]
Total	1,640	30,092 (18.4)	16,987 (56.5)	13,005 (43.2)	4,564 (15.2)

[a~d] Within the same chart, values with different superscripts differed significantly ($p<0.05$).

Table 2. Developmental rate of follicular oocytes in culture media with or without serum

Media	No. of oocytes	No. (%) of embryos development to			
		≥2-cell	8-cell	Blastocyst	No. of Grade 1, 2 (%)
Serum	578	283 (49.0)[a]	264 (45.7)	98 (17.0)	34 (34.7)[a]
Serum free	496	282 (56.9)[b]	218 (44.0)	74 (14.9)	41 (55.4)[b]
Serum+Serum free	702	393 (56.0)[b]	330 (47.0)	119 (17.0)	50 (42.0)[ab]

[a,b] Within the same chart, values with different superscripts differed significantly ($p<0.05$).

Table 3. The effect of meat yield grade on the number of oocytes recovered and their subsequent cleavage and development to blastocyst in KNCs

Meat yield	No. of examined ovary	No. of aspirated oocytes (per ovary)	No. (%) of embryos development to		
			≥2-cell	8-cell	Blastocyst
A	555	10,230 (18.4)	5,800 (56.7)[a]	4,398 (43.0)[a]	1,568 (15.3)
B	926	15,753 (17.0)	9,253 (58.7)[b]	7,217 (45.8)[b]	2,452 (15.6)
C	280	5,055 (18.1)	2,872 (56.8)[a]	2,203 (43.6)[a]	735 (14.5)

[a,b] Within the same chart, values with different superscripts differed significantly ($p<0.05$).

Table 4. The effect of meat quality grade on the number of oocytes recovered and their subsequent cleavage and development to blastocysts in KNCs

Meat quality	No. of examined ovary	No. of aspirated oocytes (per ovary)	No. (%) of embryos development to		
			≥2-cell	8-cell	Blastocyst
1++	139	2,639 (19.98)	1,482 (56.2)[a,d]	1,058 (40.1)[a]	270 (10.2)[a]
1+	209	3,949 (18.9)	2,279 (57.7)[a]	1,746 (44.2)[b]	485 (12.3)[b]
1	613	10,369 (16.9)	5,787 (55.8)[d]	4,454 (43.0)[b]	1,403 (13.5)[c]
2	612	10,770 (17.6)	6,333 (58.8)[b]	4,963 (46.1)[c]	1,954 (18.1)[d]
3	188	3,311 (17.6)	2,044 (61.7)[c]	1,597 (48.2)[d]	643 (19.4)[d]

[a~d] Within the same chart, values with different superscripts differed significantly ($p<0.05$).

between groups was not significant. Oocytes from animals with the lowest meat quality (grade 3) had higher rates of development to the two- and eight-cell stages (61.7 and 48.2%, respectively) and blastocyst stage (19.4%) compared to other groups ($p<0.05$ for all three stages).

Pregnancy rates of KNC IVM/IVF oocytes according to various conditions. The pregnancy and abortion rates of follicular oocytes after transfer according to various culture media conditions are shown in Fig. 1. The pregnancy rate with oocytes cultured in media with serum, serum free media, and combined media was 58.3, 59.1, and 59.2% respectively; there was no significant difference between the groups. The abortion rate

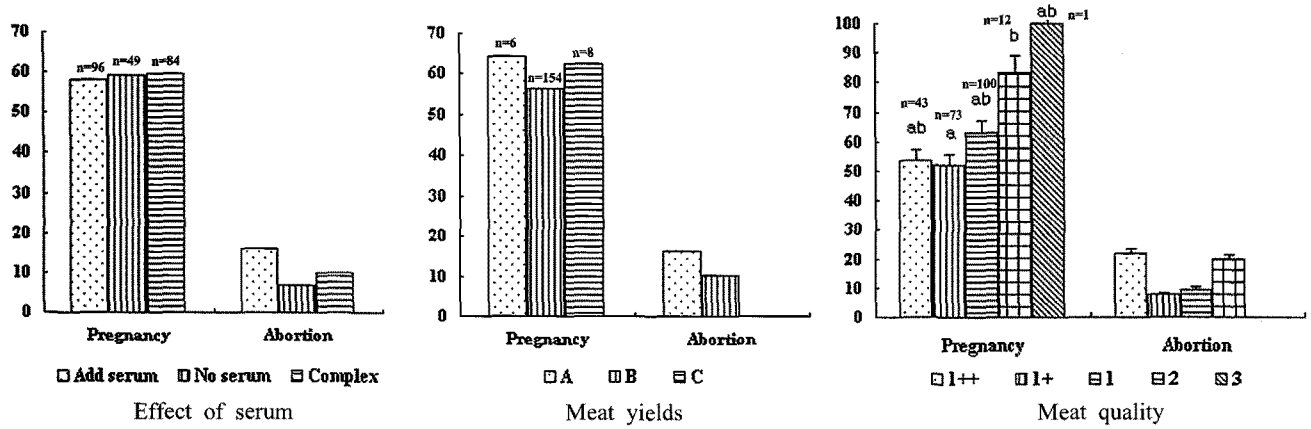

Fig. 1. Pregnancy and abortion rates after transfer of *in vitro* produced KNC embryos (*n*=total recipients).
[a~b] Within the same chart, values with different superscripts differed significantly ($p<0.05$).

was 6.7 to 16.1%, and was also not different between groups. *In vitro* embryos derived from ovaries of meat yield grade A animals tended towards a higher pregnancy rate (64.2%) than embryos derived from meat yield grade B cows (56.5%), but the difference was not significant. When embryos produced from fertilized oocytes of low meat quality KNC (grade 3) were transferred, the pregnancy rate was higher ($p<0.05$) than with other meat quality grades. There was no difference in abortion rate based on meat yield or meat quality grade.

DISCUSSION

Typically, 3.5~8.9 follicular oocytes are recovered during aspiration of *in vivo* oocytes (Gibbons *et al.*, 1994; Looney *et al.*, 1994; Bungartz *et al.*, 1995; Hasler *et al.*, 1995; Goodhand *et al.*, 1999). Mermillod *et al.* (1992) collected an average of 14.1 follicular oocytes per slaughtered cow during follicular aspiration, but we recovered an average of 18.8 follicular oocytes per cow. There is often intense individual variation in the recovery rate and grade of *in vivo*-derived follicular oocytes compared to slaughterhouse-derived follicular oocytes. Recovery of follicular oocytes from slaughtered cow ovaries is also easier and more efficient (De Roover *et al.*, 1997).

The developmental rate of recovered *in vivo*-derived follicular oocytes to blastocysts is 10~20% (Hanenberg *et al.*, 1997; Duszewska *et al.*, 2000), with a further blastocyst developmental rate of 37.1%. Izadyar *et al.* (1996) and Kajihara *et al.* (1990) reported developmental rates of slaughterhouse-derived follicular oocytes to blastocysts of 28.2 and 32%, respectively. In contrast, Mermillod *et al.* (1992) described a developmental rate to blastocysts from individual cultures of only 9.5%. Our rate of development to blastocysts was also somewhat lower at 15.2%, possibly due to the use of serum in the medium for IVF of follicular oocytes. Moreover, the use of serum-free media yielded a higher cell number and developmental rate than serum-supplemented medium. However, the serum-free media showed the developmental rate of significant good grade embryo.

Oocytes from KNC of meat yield grade B had a higher developmental rate to the two- and eight-cell stages than KNC of grades A and C ($p<0.05$), but there was no significant difference in the blastocyst developmental rate based on meat yield grade. Although there was no difference in the number of recovered follicular oocytes per ovary based on meat quality grade of the source cow, oocytes from grade 3 KNC (the lowest grade of meat quality) developed at a higher rate ($p<0.05$). Meat quality grade is an important economic characteristic that is highly dependent on the marbling score. From the perspective of reproductive physiology, however, the fat content may have a negative effect on oocyte development.

Until now, oocytes have been randomly aspirated from slaughtered cows, combined into mixed cultures, and transferred. According to this study, the transfer of blastocysts from mixed cultures has a high possibility of decreasing meat quality, because oocytes harvested from low quality meat performed the best in culture. Further study is needed to improve developmental competence and pregnancy rate through the individual culture of follicular oocytes from the ovaries of cows with good meat quality grades. This approach may be economically beneficial for the establishment of a KNC reproduction system. The quality and condition of the recipient cow, operator technique, and many other factors can influence the pregnancy rate after ET. *In vivo*-produced embryos also have a higher pregnancy rate than *in vitro*-produced embryos (Numabe *et al.*, 2000), which can be affected by embryo freezing (Hasler, 2001) number of transferred embryos (Numabe *et al.*, 2000), blastocyst grade (Linder and Wright 1983; Numabe *et al.*, 2000) and blastocyst formation period (Numabe *et al.*, 2000).

We found no significant difference among pregnancy rates after ET according to serum-containing or serum-free oocyte culture media and meat yield grade of the source animal. A significantly higher pregnancy rate was observed when the transferred embryos came from oocytes that were recovered from the ovaries of low meat quality grade cows. In terms of abortion rates, 20~30% of *in vitro*-derived embryos spontaneously aborted, which was much higher (Sakaguchi *et al.*, 2002) than reported abortion rates from *in vivo*-derived embryos (below 8%) and AI (below 5%). The rate of early embryonic death is higher for *in vitro*-produced embryos (McEvoy *et al.*, 1995), possibly due to cell number (Park *et al.*, 2005) or an increase of abnormal chromosomes, among other factors. In this study, however, there was no particular difference in abortion rate according to serum-containing or serum-free oocyte culture media, meat yield grade or meat quality grade.

This study demonstrates that it is possible to produce and transfer individual embryos derived *in vitro* from follicular oocytes collected from ovaries with a known female genetic heritage, ultimately facilitating the selection of desirable characteristics. This economic individual culture system can be used to restore and improve genetic resources in KNC, and represents a valuable tool for measuring the genetic competence of

heritable characteristics.

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