Production and Transfer of IVF Embryos from Individual Inhibin-Immunized Cows by Ultrasound-Guided Transvaginal Follicular Aspiration

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ABSTRACT. Ultrasound-guided transvaginal follicular aspiration (UTA) was performed on 8 multiparous Japanese Black cows which had not been successful in a conventional superovulation program. These cows were actively immunized with a synthetic peptide replica of porcine inhibin αN1-26 (pINH) conjugated with rabbit serum albumin (RSA) using Freund's complete adjuvant. Booster injections of the peptide were given at 6, 10, 14 and 20 wk after the primary injection. Twelve follicular aspirations were performed between 10 and 33 wk after the primary injection. Cumulus-oocyte complexes (COCs) were aspirated from follicles ≥ 2 mm in diameter. The recovered COCs were classified into 5 categories according to the surrounding cumulus cells: Grade 1 (> 4 layers), Grade 2 (3 to 4 layers), Grade 3 (1 to 2 layers), Grade 4 (denuded) and Grade 5 (expanded and fluffy cumulus cell mass or degenerated ooplasm). The recovered COCs available for *in vitro* fertilization (IVF; Grades 1, 2, 3 and 4) were matured with granulosa cells (1×10^6 cells/m*l*) for 20 to 23 hr, fertilized with frozen-thawed spermatozoa, and cultured *in vitro*. At day 7 after insemination, a portion of the embryos were transferred to Holstein heifers. There were great variations among donors in the number of recovered COCs and in the numbers of COCs in each grade, and also in the developmental ability of fertilized COCs. IVF procedures were carried out on 685 oocytes and produced 120 embryos (17.5%). The transfer of 36 fresh IVF embryos resulted in 20 pregnancies (55.6%) at 60 days after the transfer. This study has demonstrated first, that the number of COCs collected from individual inhibin-immunized cows by UTA greatly varies among donors. Second, recovered COCs vary greatly in the classification of the morphological categories of surrounding cumulus cell mass among donors and had different developmental abilities when they were cultured after fertilization *in vitro*. Third, the production of pregnancy in previously non-productive problem donors is possible using the UTA technique. — KEY WORDS: bovine, embryo transfer, IVF, pregnancy, ultrasound.

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Recently, ultrasound-guided transvaginal follicular aspiration (UTA) has been developed to collect cumulus-oocyte complexes (COCs) from living animals and has been applied to *in vitro* production of bovine embryos [6, 7, 12, 14]. Its utilization in cases of clinical cattle infertility has enabled problem donors to again become productive, and the techniques are valuable for the advancement of genetically superior dairy and beef cattle [7, 12]. Therefore, research has been directed toward collecting as many COCs as possible from cattle [18]. Hasler *et al.* [7] and Kruip *et al.* [11] have shown wide variations among non-treated cows in the number of COCs and in the developmental ability of COCs that were collected using UTA. However, these reports have not shown any relation between developmental ability of COCs and surrounding cumulus cells. A general problem with this follicular aspiration method is that the length of the needle and tubing and vacuum pressure used for UTA tend to remove a portion of the cumulus cells from the COCs [10, 12]. Surrounding cumulus cells have an essential role in cytoplasmic maturation and subsequent development *in vitro* [3, 17]. The developmental ability of COCs following IVM-IVF decreases with a reduction in the surrounding oocyte cumulus cell mass [9, 17]. On the other hand, our previous report demonstrated that active immunization of cattle against inhibin could enhance ovarian follicular development and the number of COCs collected by UTA [10]. Therefore, the primary purpose of this study was to test the influence of active immunization against inhibin on the variations among cows in the number of COCs, the morphological quality of COCs and the developmental ability of COCs collected using UTA.

This study was undertaken 1) to determine the variations of inhibin immunized cows on the number and the morphological quality of recovered COCs, and the developmental ability of COCs following IVM-IVF in each donor, and 2) to examine the number of embryos obtained from each infertile donor, and the pregnancy rate following transfer.

MATERIALS AND METHODS

Animals and treatments: Eight multiparous Japanese Black cows (440 to 520 kg, 4 to 8 years old) had been classified as infertile because of their failure to produce a viable embryo in a conventional superovulation program. The mean number of embryos produced by the 8 donors in 31 superovulatory treatments was 0.5 ± 0.2 (SEM). There were 23 superovulatory treatments (74%) that yielded no embryos. The donors were actively immunized as described in the previous report [10] with a synthetic peptide replica (Peptide Institute Inc., Osaka, Japan) of the amino acid sequence from position 1 to position 26 (numbering from the N-terminal end) of the α-subunit of porcine inhibin (pINH) conjugated with rabbit serum albumin (RSA) using Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A) containing 0.5 mg/m*l* heat-inactivated tuberculosis. The pINH: RSA weight ratio after conjugation was approximately 1:2. The amounts of pINH in pINH-

RSA for primary immunization and for boosters 1 to 4 were as follows: primary–1.0 mg pINH in 2.0 m*l* saline emulsified in 2.0 m*l* Freund's complete adjuvant; booster 1 to 4–0.5 mg pINH were given at 6, 10, 14 and 20 wk after the primary injection. All injections were given subcutaneously at three sites in the neck or shoulder.

Follicular aspiration: Aspiration was performed at 10, 11, 12, 13, 14, 15, 16, 17, 21, 25, 29 and 33 wk after the primary injection of a synthetic peptide replica of pINH by UTA as described in the previous reports [9, 10]. Briefly, follicular aspiration was conducted with an Aloka SSD 650 ultrasound monitor and a 5-MHz convex array transducer (Aloka, Co., Ltd. Tokyo, Japan) attached to a specially designed puncturing device (Chuck Boland Med., TX, U.S.A) with a 17-gauge stainless steel needle guide. Follicles ≥ 2 mm in diameter were aspirated with vacuum pressure (100 mm Hg) applied through the stainless steel needle (59 cm in length) into a 50-m*l* centrifuge tube via 100 cm of teflon tubing. PBS containing 80 μg/m*l* heparin (Mochida, Co., Ltd. Tokyo, Japan) was used for rinsing the needle and tubing. Recovered COCs were classified into 5 categories according to the surrounding cumulus cells [9, 12]: Grade 1 (> 4 layers), Grade 2 (3–4 layers), Grade 3 (1–2 layers), Grade 4 (denuded), and Grade 5 (expanded and fluffy cumulus cell mass or a degenerated ooplasm). It was considered that the COCs of Grades 1, 2, 3, and 4 were available for IVF [9].

In vitro maturation: The COCs were washed three times with maturation medium, 25 mM HEPES-buffered TCM-199 with Earl's salts (GIBCO BRL, Life Technology Inc., NY, U.S.A.) with 5% (v/v) heat-inactivated CALF SUPREME™ serum (CS; GIBCO BRL). They were then transferred to 700 μ*l* of maturation medium covered with warm mineral oil (E.R.SQUIBB, & Sons Inc., Princeton, NJ, U.S.A.) in 4-well culture plates (Nunc Inc., Roskilde, Denmark), and co-cultured with granulosa cells (1×10^6 cells/m*l*) at 39°C in an atmosphere of 5% CO_2 in air for 20 to 23 hr. The COCs of Grade 5 were excluded from IVM.

In vitro fertilization: Spermatozoa were treated as described by Funahashi *et al.* [5]. Briefly, commercially prepared frozen semen from one Japanese Black bull was thawed in a water bath at 35°C and washed twice with BSA and glucose-free modified BO [2] medium supplemented with 10 mM caffeine-sodium benzoate (C-4144, Sigma Chemical Co., St Louis, MO, U.S.A.). Fifty microliters of the sperm suspension diluted to $5–6 \times 10^6$ cells/m*l* were added to 50 μ*l* of glucose-free modified BO fertilization medium supplemented with 20 mg/m*l* BSA and 20 μg/m*l* heparin (H-9399, Sigma) containing COCs. Therefore, the final concentration of spermatozoa, caffeine, BSA and heparin during insemination was 2.5 to 3×10^6 cells/m*l*, 5 mM, 10 mg/m*l* and 10 μg/m*l*, respectively. After the co-culture of COCs with spermatozoa at 39°C in an atmosphere of 5% CO_2 in air for 6 hr, the COCs were transferred to 700 μ*l* of CR1aa [15] medium containing 2% CS and covered with warm mineral oil in 4-well culture plates, then cultured at 39°C in an atmosphere of 5% CO_2 in air for 7 days. The contaminated oocytes during aspiration were excluded from IVF.

Assessment of in vitro development and embryo transfer: The incidence of COCs that developed to the morula/blastocyst stages was determined at day 7 after insemination. At day 7 after insemination, a portion of the embryos was transferred to holstein heifers (one embryo per recipient) that were in days 6 to 8 of the estrous cycle. Pregnancy of the recipients was determined twice by transrectal ultrasonography at 30 and 60 days after the transfer.

Statistical analysis: Statistical analysis on the number of recovered COCs was carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test. The significance of the differences on categories of recovered COCs and inseminated COCs among donors were examined by non-parametric analysis. The developmental ability and the pregnancy rate were analyzed by a chi-square test. Probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

As shown in Table 1, there was a great deal of variation among the donors in the number of recovered COCs and in the numbers of COCs in each grade. There were significant differences ($P<0.05$) among donors in the number of recovered COCs and the mean category of COCs. Although the number of recovered COCs from each donor varied, only 1 collection (1.0%) yielded no oocytes (data not shown).

As shown in Table 2, there were great variations among donors in the mean category of COCs which were used for IVF, and great variations in the developmental abilities of COCs that had developed to the morula/blastocyst stages. The mean category score of donor G (2.0 ± 0.1) was significantly smaller than that of donor C (2.4 ± 0.1) ($P<0.05$). However, the incidence of embryos developing to the morula/blastocyst stages was lower for donor G than donor C (9.7% versus 27.8%; $P<0.05$). The transfers of embryos were successful in producing pregnancies at 60 days after the transfer in all donors except A and H.

Neither the developmental stage of the embryos nor the time of the recipient's estrous cycle had an effect on the pregnancy rate (data not shown).

DISCUSSION

In the present study, there were great variations in the number of recovered COCs and in the numbers of COCs in each grade among donors. Previous studies have reported variations in the number of COCs recovered from the ovaries of individual heifers at a slaughterhouse (range, 4–66) [5] and in the mean number of COCs per collection collected from non-treated cows by UTA (range, 1.6–10.0) [9]. To our knowledge, this is the first report of variations in the number of recovered COCs and in the numbers of COCs in each grade collected from individual inhibin-immunized

Table 1. Variations among individual cows in the number of recovered COCs and classified COCs collected by ultrasound-guided transvaginal aspiration

Donor	No. of recovered COCs (N_1)	No. of classified COCs[a] (N_2) (rate of N_2:N_1)					Mean category of COCs
		Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	
A	94 (7.8 ± 1.4)[b]	13 (14)	27 (29)	18 (19)	16 (17)	20 (21)	3.0 ± 0.1[b]
B	81 (6.8 ± 1.1)[b,c]	20 (25)	31 (38)	12 (15)	3 (4)	15 (19)	2.5 ± 0.2[d,e]
C	65 (5.4 ± 1.1)[b,c]	7 (11)	29 (45)	9 (14)	9 (14)	11 (17)	2.8 ± 0.2[b,e]
D	116 (9.7 ± 1.6)[b]	15 (13)	45 (39)	19 (16)	18 (16)	19 (16)	2.8 ± 0.1[b,d,f]
E	279 (23.3 ± 3.1)[d]	39 (14)	67 (24)	80 (29)	44 (16)	49 (18)	3.0 ± 0.1[b]
F	107 (8.9 ± 1.2)[b]	21 (20)	37 (35)	23 (21)	11 (10)	15 (14)	2.6 ± 0.1[c,e,f]
G	92 (7.7 ± 1.3)[b]	30 (33)	25 (27)	16 (17)	6 (7)	15 (16)	2.5 ± 0.1[e]
H	37 (3.1 ± 0.7)[c]	5 (14)	10 (27)	9 (24)	5 (14)	8 (22)	3.0 ± 0.2[b,c]

Experiments were repeated twelve times. Numbers and categories of COCs are expressed as the mean ± SEM. [a] Classification of COCs: Grade 1, >4 layers of cumulus cells; Grade 2, 3 to 4 layers of cumulus cells; Grade 3, 1 to 2 layers of cumulus cells; Grade 4, denuded; and Grade 5, expanded and fluffy cumulus cell mass or degenerated ooplasm. [b,c,d,e,f] Different superscripts within columns denote significant differences ($P<0.05$).

Table 2. Variations among individual cows in *in vitro* development of COCs following IVM-IVF and pregnancy rate after transfer of embryos

Donor	No. of inseminated COCs	Mean category of inseminated COCs[a]	No. (%) of embryos (≥ morula)	No. of transfers	No. (%) of pregnancies after the transfer	
					Day 30	Day 60
A	74	2.5 ± 0.1[b,c]	11 (14.9)[b,c]	–	–	–
B	57	2.0 ± 0.1[d,e]	18 (31.6)[d]	3	2 (67)	2 (67)
C	54	2.4 ± 0.1[b,c,d]	15 (27.8)[c,d]	7	4 (57)	3 (43)
D	97	2.4 ± 0.1[b,c]	19 (19.6)[b,d]	8	4 (50)	4 (50)
E	230	2.6 ± 0.1[b]	29 (12.6)[b]	8	5 (63)	4 (50)
F	83	2.3 ± 0.1[c,d,e]	19 (22.9)[c,d]	7	6 (86)	5 (71)
G	72	2.0 ± 0.1[e]	7 (9.7)[b]	3	2 (67)	2 (67)
H	18	2.7 ± 0.2[b,c]	2 (11.1)[b,d]	–	–	–

Numbers and categories are expressed as the mean ± SEM. [a] Classification of cumulus-oocyte complexes (COCs): Grade 1, > 4 layers of cumulus cells; Grade 2, 3 to 4 layers of cumulus cells; Grade 3, 1 to 2 layers of cumulus cells; and Grade 4, denuded. [b,c,d,e] Different superscripts within columns denote significant differences ($P<0.05$).

cows by UTA. It is known that a portion of cumulus cells surrounding the COCs collected by UTA tend to be removed by the long needle and tubing and vacuum pressure [10, 12]. In the present results, the variation in the category of recovered COCs among donors cannot be completely explained by these physical factors. It seems that the individual differences may be due to other unknown factors. Our results confirm that repeated UTA for the collection of immature COCs from infertile donors using active immunization against inhibin is possible during a period of at least 33 weeks. Therefore, under the present immunization, it is possible that we can harvest greater numbers of COCs by practicing UTA through this period on a once- or twice-per-week basis [6, 7, 16, 18].

The developmental ability of oocytes following IVM-IVF decreases with reduction in the surrounding oocyte cumulus cell mass [9, 17]. Therefore, enhancing the developmental ability of COCs containing fewer layers of cumulus cells may increase embryo production from a limited number of COCs derived from infertile donors. Our previous report [9] demonstrated that 4 or more layers of cumulus cells surrounding the oocytes is optimum for development to the blastocyst stage, although granulosa cell supplementation can improve the developmental ability of COCs containing fewer layers of cumulus cells. That report also showed that the ability of COCs in Grades 1 and 2 to develop to the blastocyst stage was higher than those in Grades 3 and 4 under the present culture conditions. It has been reported that the developmental ability of COCs was affected by not only the morphological categories of surrounding cumulus cell mass but also the quality of the ooplasm [1, 8]. In addition, developmental ability might be affected by wide ranges in the numbers of cultured COCs in each well. Therefore, further studies are needed to classify collected COCs not only according to the surrounding cumulus cell mass but also according to the quality of ooplasm for determining the developmental ability of each cow. On the other hand, Funahashi *et al.* [5] have reported that the COCs aspirated from slaughterhouse ovaries of different heifers had the same developmental capacity following IVM-IVF. However, our results do not contradict their report, because of a difference in experimental designs. They collected

COCs only at a single time point and cultured only COCs with a dense layer of cumulus cell mass. In contrast, we aspirated repeatedly from donors during pINH immunization using the UTA technique and cultured all COCs containing fewer layers of cumulus cells, except for COCs with an expanded and fluffy cumulus cell mass or a degenerated ooplasm.

The overall pregnancy rate of 55.6% (20/36) for fresh IVF embryos in the present study was similar to that reported in tow previous studies [7, 13]. In agreement with [7], it seems that there is no difference in pregnancy rate among donors. It is difficult to evaluate the viability of embryos from our transfer results, because there were only a small number of transfers. Data from a large number of transfers are required to evaluate the viability of bovine IVF embryos.

This study has demonstrated first, that active inhibin immunization could not reduce the variation among donors in the number of COCs collected by UTA. Second, the recovered COCs vary greatly among donors in their morphology relating to the surrounding cell mass and in their developmental ability when they were cultured after fertilization *in vitro*. Third, the production of pregnancy from previously non-productive problem donors is possible using the UTA technique.

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