NOTE ***Theriogenology***

Relationship between the Responsiveness to Multiple-ovulation Treatment and the Number of Bovine Oocytes Collected by Transvaginal Follicle Aspiration

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ABSTRACT. To characterize factors affecting the number of bovine oocytes recovered transvaginally, a regression analysis was performed between the responsiveness to multiple-ovulation treatment and the number of oocytes recovered transvaginally. The number of embryos recovered following multiple-ovulation treatment and the number of oocytes recovered transvaginally increased when the number of follicles to be aspirated transvaginally increased ($P<0.05$, $P<0.01$). The number of cumulus-oocyte complexes recovered transvaginally also increased when the number of oocytes to be aspirated transvaginally increased ($P<0.001$). However, the number of viable embryos that recovered following multiple-ovulation treatment had no relation to the number of cumulus-oocyte complexes recovered transvaginally. These results suggested that more oocytes can be recovered from donors that have a high responsiveness to multiple-ovulation treatment.—KEY WORDS: bovine oocytes, cattle, transvaginal aspiration.

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Ultrasound-guided transvaginal aspiration of bovine oocytes has been proven to be a successful and repeatable technique for the production of embryos and calves from donors with normal estrous cycles [10, 12], problem donors [14] and pregnant donors [15, 18]. Therefore, transvaginal oocyte aspiration followed by *in vitro* maturation, *in vitro* fertilization (IVF) and *in vitro* culture has been thought to be a potential alternative to multiple-ovulation treatment. However, the factors affecting the number of oocytes recovered transvaginally are not as well understood as the factors affecting the responsiveness of cows to multiple-ovulation treatment. Recently, the responsiveness of cows to multiple-ovulation has been shown to be partly dependent on the number of small follicles (2- to 3-mm: [11] or 3- to 6-mm diameter: [17]) at the onset of the treatment. Furthermore, in our previous study, the proportion of follicles at 3 to 5 mm in diameter per aspirated follicle was more than 90% [8]. Thus, it is thought that more oocytes can be recovered from donors with a higher responsiveness to multiple-ovulation treatment. If this idea is true, we can select donor cows that have a high potential to produce more embryos following transvaginal oocyte collection and IVF according to their responsiveness to the multiple-ovulation treatment. To assess this hypothesis, we examined the relationship between the responsiveness of cows to multiple-ovulation treatment and the number of oocytes collected by transvaginal follicle aspiration.

The donor cows used in the present study consisted of 11 Japanese-black-beef cows and 1 Holstein cow, ranging from 2 to 17 years old. Multiple-ovulation treatment was started at Day 9 to 11 (day of estrus=Day 0 of the estrous cycle). Multiple-ovulation treatment for a cow consisted of twice-daily intramuscular injections of descending doses of follicle stimulating hormone (FSH: Antrin; Denka, Tokyo, Japan) for 3 or 4 days, starting with 3 to 6 AU, for a total dose of 18 to 30 AU. Cows received either 75 mg dinoprost (Pronalgon®F, Amersham Pharmacia Biotech, Uppsala, Sweden) or 0.5 mg chloprostenol (Estrumate, Sumitomo Seiyaku, Osaka, Japan) on day 3 of FSH treatment. Donors were artificially inseminated 1 time, or 2 times at a 12 hr interval, with frozen-thawed semen following the onset of estrus.

Embryos were recovered 7 days after estrus as described by Hasler *et al.* [9]. Briefly, using 2 way balloon catheters (Fujihira Industries Co., Ltd., Tokyo, Japan), each uterine horn was separately flushed with a total of 500 m*l* modified Dulbecco's phosphate buffered saline (PBS) or Ringer's solution, containing heat-inactivated 1% newborn calf serum (Gibco BRL, Life Technologies, Inc., Grand Island, NY, U.S.A.). The recovered solution was rinsed through an EM-con filter (Immuno Systems Inc., WI, U.S.A.) with sterilized PBS. All embryos transferred into PBS containing 10% heat-inactivated newborn calf serum were evaluated under a stereomicroscope (× 60) and classified according to the manual of the International Embryo Transfer Society [19]. Embryos classified as code 1 (excellent or good) or code 2 (fair) were regarded as viable embryos. Embryo collection was carried out from December 1996 to January 1999, and replicated 3 times.

Follicle aspiration was carried out as previously described [7]. Briefly, 3- to 10-mm follicles were aspirated from the donors used in the multiple-ovulation treatment, within 1 year after the last multiple-ovulation treatment. Oocytes were collected once a week consecutively using a 5.0 MHz probe (UST-588U-5, Aloka, Tokyo, Japan) attached to an echo camera (SSD-500; Aloka) with an oocyte aspiration system consisting of an 18-gauge, 7-cm-long disposable needle (Inner diameter: 0.9 mm, Fujihira Industries Co., Tokyo, Japan), an aspiration line (a 60-cm-

Table 1. The numbers of total and viable embryos recovered following multiple-ovulation, and the numbers of oocytes and group 1 oocytes recovered by transvaginal follicle aspiration

Cow	Multiple-ovulation		Transvaginal follicle aspiration			
	Mean number per treatment ± SEM			Mean number per treatment ± SEM		
	Embryos	Viable embryos	Number of trials	Follicles aspirated	Oocytes	Group 1 oocytes*
A	10.0 ± 2.0	6.0 ± 0.6	5	7.4 ± 1.0	2.4 ± 0.9	1.8 ± 0.4
B	13.0 ± 2.3	7.3 ± 3.8	5	12.2 ± 1.0	3.8 ± 1.2	2.8 ± 1.1
C	9.3 ± 5.5	3.0 ± 3.0	5	9.6 ± 1.5	3.6 ± 0.7	2.8 ± 0.8
D	4.0 ± 2.1	0	5	8.0 ± 0.7	1.6 ± 0.5	1.0 ± 0.4
E	17.0 ± 8.9	7.3 ± 3.8	5	12.0 ± 2.4	6.6 ± 1.7	6.0 ± 1.7
F	7.3 ± 2.0	4.3 ± 2.4	4	5.3 ± 0.6	2.3 ± 0.9	1.5 ± 0.5
G	10.7 ± 3.3	0	3	12.3 ± 1.3	8.7 ± 0.9	6.0 ± 1.5
H	10.7 ± 0.7	0	3	14.0 ± 1.0	10.3 ± 0.9	6.3 ± 0.9
I	3.3 ± 1.2	0	3	5.7 ± 0.3	1.0 ± 0.6	1.0 ± 0.6
J	20.3 ± 2.2	13.7 ± 0.9	2	12.0 ± 2.0	8.0 ± 1.0	7.0 ± 1.0
K	8.3 ± 3.3	3.3 ± 2.8	2	3.0 ± 1.0	2.0 ± 0	1.0 ± 0
L	13.7 ± 7.0	2.3 ± 1.2	3	13.0 ± 2.1	6.7 ± 0.9	5.3 ± 1.2

* Oocytes had at least 1 layer of cumulus cells.

long Teflon tube attached to a 120-cm-long tube with a silicon bung, Fujihira Industries Co.) and a vacuum pump (K-MAR-5000; Cook, Brisbane, Australia). The vacuum was set at 40-mmHg (corresponding to a flow rate of 516-μl/sec). The needle was rinsed with flushing buffer. The flushing buffer consisted of 25-mM HEPES-buffered synthetic oviduct fluid (without glucose, BSA or $NaHCO_3$), containing heat-inactivated 1% newborn calf serum and 10 units/m*l* heparin (H-3393; Sigma Chemical Co., St. Louis, MO, U.S.A.). The aspirated follicular fluid and oocytes were pooled in a 50-m*l* tube (Falcon 2098; Beckton Dickinson Labware, NJ, U.S.A.) with flushing buffer. Termination of aspiration of follicular fluid was determined by the disappearance of follicles on the monitor. Transvaginal oocyte collection was carried out from May 1997 to February 1999, and replicated 2 to 5 times.

The recovered oocytes were divided into 2 groups according to the presence and the number of cumulus cell layers surrounding the oocytes. Oocytes classified as cumulus-oocyte complexes (group 1 oocytes) had at least 1 layer of cumulus cells and completely or partially denuded oocytes; all other oocytes, i.e., those with expanded cumulus cells or degenerated oocytes were classified as group 2 oocytes. Oocytes surrounded by only 1 cumulus cell layer have been shown to have the same developmental competence as oocytes with 3 or more cell layers when cumulus cells are added during *in vitro* maturation [6, 13].

The average numbers of total and viable embryos recovered following the multiple-ovulation treatment are shown in Table 1. Table 1 also shows the average numbers of follicles aspirated, and the average numbers of oocytes and group 1 oocytes recovered by transvaginal follicle aspiration. We assessed the relationship between (1) the number of embryos recovered following the multiple-ovulation treatment and the number of follicles aspirated transvaginally, (2) the number of follicles aspirated and the number of oocytes recovered transvaginally, (3) the number of oocytes and the number of group 1 oocytes recovered transvaginally, and (4) the number of viable embryos recovered following the multiple-ovulation treatment and the number of group 1 oocytes recovered transvaginally. These relationships were evaluated using a regression analysis (StatView program 5.0; SAS Institute Inc., Cary, NC, U.S.A.). The applied model is as follows. The number of follicles aspirated, the number of oocytes recovered transvaginally and the number of group 1 oocytes recovered transvaginally were each expressed as a linear function of one of four variables, resulting in a total of 12 equations of the form $f(x) = a_0 + a_1x$. The four variables x are (1) the number of total embryos recovered following multiple-ovulation, (2) the number of follicles aspirated, (3) the number of oocytes recovered by transvaginal follicle aspiration, and (4) the number of viable embryos recovered following multiple-ovulation.

The number of follicles aspirated transvaginally increased with increasing number of total embryos recovered following the multiple-ovulation ($f(x)=4.516 + 0.472x$, $P=0.0229$, $r^2=0.419$; Fig. 1). The number of oocytes recovered transvaginally increased with increasing number of follicles aspirated transvaginally ($f(x)= -2.35 + 0.744x$, $P=0.0005$, $r^2=0.714$; Fig. 2). Moreover, the number of group 1 oocytes recovered transvaginally increased with increasing number of oocytes recovered transvaginally ($f(x)=0.087 + 0.726x$, $P<0.0001$, $r^2=0.923$; Fig. 3). However, the number of viable embryos recovered following multiple-ovulation did not affect the number of group 1 oocytes recovered transvaginally ($P=0.29$).

The results of the present study indicated that there were strong relationships among the numbers of follicles aspirated, the number of oocytes recovered transvaginally and the number of group 1 oocytes recovered transvaginally. Thus, these results suggested that the technical level used in this study was reliable and that the number of group 1 oocytes recovered transvaginally is

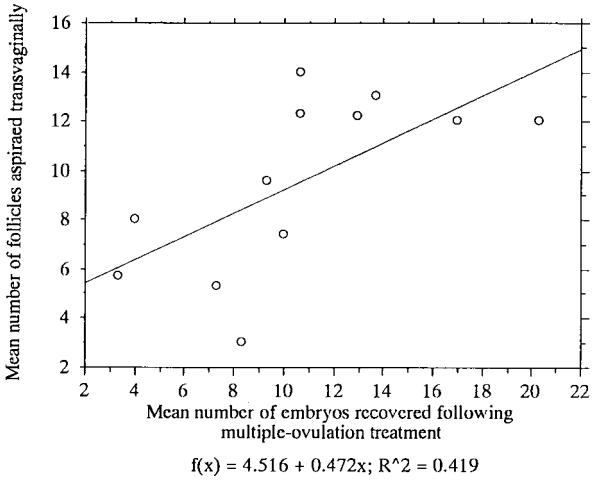

Fig. 1. Relationship between the number of total embryos recovered following multiple-ovulation and of follicles aspirated transvaginally.

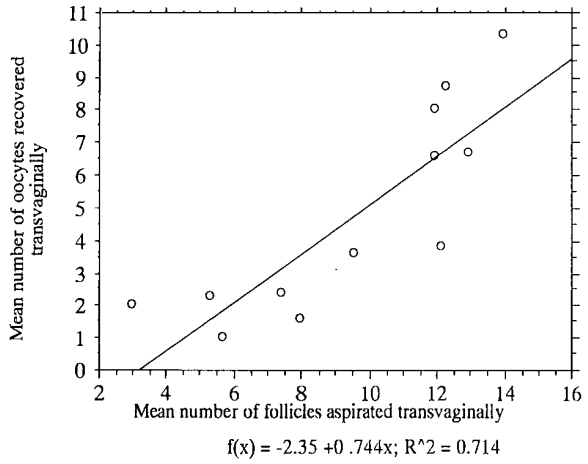

Fig. 2. Relationship between the number of follicles aspirated and of oocytes recovered transvaginally.

dependent on the number of aspirated follicles, as shown in a previous study [8]. From the data of the relationship between the number of embryos recovered following multiple-ovulation treatment and the number of follicles aspirated transvaginally, it was suggested that the number of follicles emerged in each estrous cycle is almost the same for at least one year in the same donor cow, and is regulated for each individual animal as suggested by Boni *et al.* [1]. Therefore, the individual differences in the number of oocytes recovered transvaginally [5] may be caused by differences in the number of emerged follicles.

The responsiveness of cows to multiple-ovulation has been shown to be partly dependent on the number of small follicles at the onset of the treatment [11, 17] and to be increased in donors with more than 10 small follicles [11]. In the present study, transvaginal oocyte collection was carried out from follicles at 3 to 10 mm in diameter and the

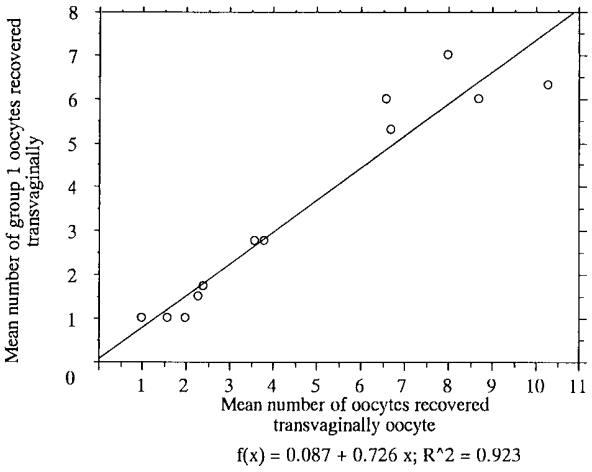

Fig. 3. Relationship between the number of oocytes and of group 1 oocytes recovered transvaginally.

proportion of follicles at 3 to 5 mm in diameter per aspirated follicle was more than 80%. Therefore, it is believed that the number of transvaginally aspirated follicles per individual increases with increasing responsiveness to multiple-ovulation treatment. Consequently, the numbers of oocytes and group 1 oocytes collected by transvaginal follicle aspiration increased with increasing number of follicles aspirated.

No relationship was detected between the number of viable embryos recovered following multiple-ovulation and the number of group 1 oocytes recovered transvaginally. The number of viable embryos recovered following the multiple ovulation is affected by various factors including the number of gonadotrophin injections per day [2], the amount of gonadotrophin administered [4, 16] and so on. Especially, it has been reported that endometritis decreased the number of viable embryos [3]. On the other hand, the number of group 1 oocytes recovered by transvaginal aspiration is mostly dependent on the number of aspirated follicles, rather than on the above-mentioned factors. Thus, it is suggested that the number of group 1 oocytes recovered by transvaginal aspiration is independent of the number of viable embryos recovered following multiple-ovulation.

In conclusion, the results of the present study indicated that more oocytes can be recovered from donors with a higher responsiveness to multiple-ovulation treatment in spite of the number of viable embryos recovered following multiple-ovulation treatment. These results suggest that transvaginal oocyte collection can be a powerful tool to produce embryos and offspring from donors that have a high responsiveness to multiple-ovulation with little potential to produce viable embryos following multiple-ovulation treatment. Thus, we can select donor cows that have the potential to produce more embryos followed by transvaginal oocyte collection and IVF technology according to their responsiveness to multiple-ovulation treatment.

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