ELSEVIER

MORPHOLOGICAL CLASSIFICATION OF THE OVARIES IN RELATION TO THE SUBSEQUENT OOCYTE QUALITY FOR IVF-PRODUCED BOVINE EMBRYOS

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Received for publication: January 21, 1998
Accepted: August 7, 1998

ABSTRACT

Although some inferences have been made regarding the morphological aspects of the ovaries in relation to the subsequent oocyte developmental competence in an in vitro system, the influence of ovarian morphology, taken as a pair, has yet to be demostrated. The present study addresses this limitation. Forty pairs of ovaries from 5 morphological classes were examined to determine whether their characteristics could influence oocyte yield and developmental competence in vitro. An ovary was designated as bearing a corpus luteum (CL) with a dominant follicle (DF) a cyst (CY) or none of these structures (NO). Thus, the paired classes considered in this study consisted of 1) CL-NO 2) CL-DF 3) CL+DF-NO 4) NO-DF and 5) NO-NO. Comparisons were made among the members of 3 subgroups CL, NO and DF.

Within the CL-subgroup, the pairs of CL-NO ovaries resulted in higher ($P<0.01$) number of oocytes, cleavage rates and blastocyst formation per ovary than in the other categories (CL+DF-NO and CL-DF), with the latter being superior ($P<0.01$) to that of CL+DF-NO in terms of cleavage rates. In the NO-subgroup, NO-CL pairs yielded higher ($P<0.01$) rates of oocyte recovery and cleavage than the NO-DF pairs, and the latter was inferior ($P<0.05$) to that of NO-NO ovaries for the 2 indices. Further, blastocyst rates from the NO-CL pairs was higher ($P<0.01$) compared with those of NO-CL+DF, NO-DF, and NO-NO groups. And, in the DF-subgroup, the DF-CL pairs gave a higher ($P<0.05$) oocyte yield and cleavage rate ($P<0.01$) than the pairs of DF-NO ovaries but not significantly different in blastocyst formation. The overall oocyte recovery, cleavage and blastocyst rates for the 5 classes were, in a decreasing order CL-NO; NO-NO; CL-DF; CL+DF-NO; and DF-NO. Our results suggest that the morphological classification of ovarian pairs could be a useful means for predicting the developmental competence of oocytes in vitro, and that the presence of a dominant follicle in either one or both ovaries of a pair has a negative effect on the IVF-produced bovine embryos.

Key words: morphology, dominant follicle, corpus luteum, estrous cycle, blastocyst

INTRODUCTION

The production of embryos by in vitro maturation (IVM), fertilization (IVF) and culture (IVC) of oocytes aspirated directly from ovarian follicles is performed for therapeutic reasons in humans, for production in domestic animals, and for experimental research in laboratory animals (25). Together with the IVF production of bovine embryos, another area of particular interest in this field is the aspiration of oocytes from a live cow by the use of ultrasound-guided equipment. The commercial value of this method over IVF lies in that it would allow breeders to produce a high number of embryos from their most valuable cows even when these animals are old, have reproductive disorders such as adhesions or blocked fallopian

Acknowledgements
The authors wish to thank the Hiroshima and Kita Kyushu abattoirs for the supply of ovaries and the Yamaguchi Prefecture Zootechnic Experimental Station for providing the superovulated cow's serum. Further thanks are extended to Drs. J. Muleya, D.D. Gunawardena and M.A. Kakar for their helpful comments on the manuscript.

0093-691X/98/$19.00
PII S0093-691X(98)00204-0

tubes, in early terms of pregnancy, not responding to induced multiple ovulations or are no longer capable of becoming pregnant (12, 13, 21).

Ultrasonographic studies have revealed the fact that ovarian follicular development during the bovine estrous cycle occurs in 2 to 3 waves (12, 16, 20) and that, during each wave, a single follicle becomes dominant whereas other follicles in the same wave regress (22, 23, 24). From this event, it is believed that the dominant follicle of ovulation exerts an inhibitory effect over the growth and development of other follicles in the cohort. Dominance therefore is a process whereby the large, potentially ovulatory follicle escapes atresia, whereas subordinate follicles undergo regression. Depending on these morphological and endocrinological aspects of the ovaries at a particular moment of the estrous cycle of a cow, various studies have been conducted to determine the reproductive performance of both in vivo- (4, 5, 11, 15, 25) and in vitro-produced bovine embryos (3, 7, 9, 17, 25).

However, regarding the in vitro-produced embryos, many of the studies conducted so far have relied heavily on individual cow performance or on a pooled classification of ovaries from an abattoir (i.e., with same morphological characteristics but not identical since the morphology of the second ovary of a pair is not known and such ovaries are basically from different sources/animals). Because intraovarian activity is mainly endocrinological in nature and occurs bilaterally rather than unilaterally, studies on the influence of ovarian morphology, taken as a pair, from an individual cow for IVF-produced embryos has not been demonstrated. That being the case, a reliable method of studying the quality of oocytes for the production of IVF embryos on an individual basis should be based on the separation of each pair of ovaries from each cow.

Therefore, the aim of the present experiment was to establish morphological classification of the ovaries and study how these morphological aspects of an ovary could influence both the quality of the recovered oocytes and their developmental capacity in vitro.

MATERIALS AND METHODS

Experimental Design

Ovaries were collected separately from Holstein cows using small perforated polythine bags and transported from the abattoir in Ringer's solution supplemented with penicillin-G (100 IU/ml) and streptomycin sulfate (0.2 µg/ml) at 30 to 32 °C within 3 h of collection. Ovaries were classified according to their morphological aspect on the basis of the presence or absence of a corpus luteum, dominant follicle and cyst (whether singularly or in a conjuncture per given ovary). An ovary bearing a corpus luteum was designated as (CL), with dominant follicle as (DF), with cyst as (CY) and the one bearing none of these structures as (NO). Thus, the following classes (in a pair) were obtained: 1) CL-NO; 2) CL-DF; 3) CL+DF-NO; 4) NO-DF; 5) NO-NO; 6) CY-CY; 7) CY-NO; 8) CY-DF; 9) DF+CL-CL; and 10) CY+CL-CY. The sign "+" indicates that the morphological characteristics shown are found within a single ovary of a pair, while the "-" symbol was used to separate the 2 ovaries in one pair. It should also be noted that the distinction between dominant follicle (DF) and cystic (CY) ovary was based mainly on size (the cystic ovary is sizewise [>25 mm in diameter] than DF [<20 mm in diameter]) and texture (CY ovary has thin, soft-crepitant wall compared with the thin and relatively hard wall of the DF). Furthermore, an ovary was considered luteal (CL) irrespective of the size of the corpus luteum.

In Vitro Maturation (IVM)

Cumulus-oocyte complexes (COCs) from follicles between 2 to 5 mm in diameter were aspirated with an 18-g needle, using modified PBS (PBS supplemented with 3% BSA) as the aspiration medium. The aspirated oocytes were separately collected and washed 3 times in a maturation medium consisting of TCM-199 (Earle's salt; Gibco, Grand Island, NY, USA) supplemented with 5% Day -7 superovulated cow serum (SCS; 17), 0.01 mg/ml FSH (Denka Pharmaceutical Co., Kawasaki, Japan) and 50 µg/mL gentamicin (Sigma Chemicals, St. Louis, MO, USA). The COCs with an intact cytoplasm and surrounded by expanded

cumulus cells over more than one-third of their surface were selected and placed into the maturation medium, covered with mineral oil (Squibb & Sons, Inc, Priceton, NJ, USA) and cultured for 21 to 22 h at 38.5 °C in 5% C O_2 in air.

In VitroFertilization (IVF) and Culture (IVC)

Frozen semen was thawed in a waterbath (37°C), then washed 2 times using 2.5 mM caffeine in Bracket and Oliphant's medium (Caff-BO), according to the standard procedure (2), by centrifugation at 500g for 5 min. The sediment was then suspended in Caff-BO supplemented with 1% BSA (Sigma) and 20 µg/mL heparin (Shimizu Pharmaceutical Co., Shimizu, Japan) to yield a final sperm concentration of 5 x 10^6 /mL. A 100µL aliquot of sperm suspension was covered with mineral oil and then preincubated for 1 h at 38.5 °C in 5% CO_2 in air. Matured oocytes from each ovary were transferred into sperm microdrops for insemination.

Five hours after insemination, the COCs were washed 3 times and transferred into fresh culture medium (TCM-199 supplemented with 5% SCS), 5 µg/mL insulin (Wako Pure Chemical Industries Ltd, Osaka, Japan) and 50 µg/mL gentamicin. Cumulus cells surrounding the embryo were removed 48 h later by repeated pipetting, while the cumulus cell layer attached to the bottom of the culture dish was used as a co-culture. The culture medium was changed every 96 h. Cleavage (2-, 4-, and 8-cell) rate was recorded starting from 48 h after fertilization (Day=0), and so were the number of cleaved embryos that had developed to the blastocyst stage on Days 7, 8 and 9 post insemination.

Data Analysis

The number of classes formed from the morphological aspect of the ovaries followed a somewhat (Figure 1) normal distribution. However, due to the wide disparity in the number of observations among them, only 5 classes were considered for analysis of oocyte yield, cleavage rate and blastocyst rate. For this, 40 pairs of ovaries were used from each morphological class, and binomial data were analyzed by Chi-square test (per pair), with the overall data compared by ANOVA among the 3 subgroups (CL, NO and DF). The means are presented as least square means ± SEM.

RESULTS

Table 1 summarizes the oocyte recovery, cleavage and blastocyst rates for the 5 ovarian morphological classes analyzed in this study. For purposes of simplicity, the oocyte yield and the cleavage and blastocyst rates were analyzed separately (as shown in Figures 2 to 4) by comparing the 3 indices above among 3 subgroups (CL, NO and DF). When the CL-bearing (CL-subgroup) pairs were compared (Figure 2), the pair of CL-NO ovaries yielded a higher ($P<0.01$) number of oocytes per ovary than that of CL-DF and CL+DF-NO pairs (9.5 ± 2.4 vs 7.9 ± 2.0 and 6.2 ± 1.9, respectively). Moreover, the latter two pairs also differed significantly ($P<0.01$).

Similarly, members in the NO-subgroup varied in accordance to the other component of the other ovary pair. Hence, NO-CL ovaries had a higher ($P<0.01$) oocyte yield than the NO-DF pair (8.3 ± 2.2 vs 6.6 ± 1.9, respectively); and the latter pair resulted in a lower ($P<0.01$) yield of oocytes than that of the NO-NO pair (8.2 ± 2.1). When the DF-CL pairs were compared with DF-NO pairs (in the DF-subgroup), the former yielded a higher ($P<0.05$) number of oocytes than the latter (6.9 ± 1.9 vs 6.0 ± 1.6). The overall oocyte yield from the 5 ovarian morphological classes, arranged in a descending order, were as follows: NO-CL; NO-NO; CL-DF; DF+CL-NO; and DF-NO.

As seen in Figure 3, the highest cleavage indice among the 3 subgroups belonged to component pair of the CL-subgroup (i.e., the CL-NO pair). Comparisons made between this CL-subgroup showed that the CL-NO pair of ovaries gave a higher ($P<0.01$) cleavage rate

than both the CL-DF and DF+CL-NO pairs (7.1 ± 1.8 vs 5.7 ± 1.8 and 4.2 ± 1.4, respectively).

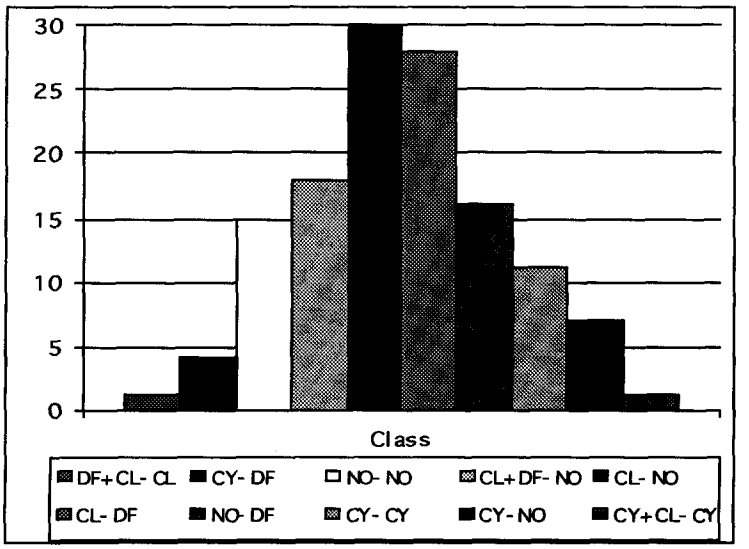


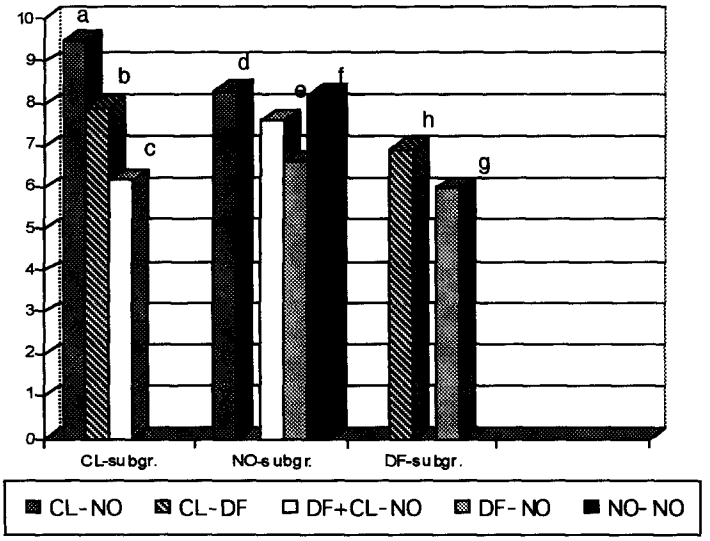
Figure 1. Frequency (distribution) of ovarian morphological classes observed in this study

Table 1. Oocyte yield and cleavage and blastocyst rates per ovary for the 5 ovarian morphological classes.

Morphological classes		Oocyte yield $\bar{x}$ ± SEM	Total	Cleavage rate $\bar{x}$ ± SEM	%	Blastocyst rate $\bar{x}$ ± SEM	%
I	NO	8.3±2.2^{d}	332	6.1±1.7^{i}	73	2.6±1.1^{u}	43
	CL	9.5±2.4^{a}	380	7.1±1.8^{i}	75	3.3±1.0^{r}	46
II	DF	6.9±1.9^{h}	276	4.9±1.6^{q}	71	1.6±0.7	33
	CL	7.9±2.0^{b}	316	5.7±1.8^{j}	72	2.0±1.0^{s}	35
III	DF+CL	6.2±1.9^{c}	248	4.2±1.4^{k}	65	1.4±0.9^{t}	33
	NO	7.6±1.8	304	5.4±1.4	71	1.6±0.7^{v}	27
IV	DF	6.0±1.6^{g}	240	4.1±1.1^{p}	68	1.4±0.7	34
	NO	6.6±1.9^{e}	263	4.5±1.5^{m}	68	1.5±0.7^{w}	33
V	NO	8.2±2.1^{f}	326	5.5±1.5^{n}	67	1.9±0.8^{x}	35
	NO	8.1±2.4	322	5.6±1.8	69	1.9±0.9	34

Oocytes:- I) a-b; a-c; b-c; d-e; and e-f ($P<0.01$).
II) g-h ($P<0.05$).
Cleavage:- I) i-j; i-k; j-k; l-m; p-q ($P<0.01$).
II) m-n ($P<0.05$).
Blastocyst:- I) r-s; r-t; u-v; u-w; u-x ($P<0.01$).
II) s-t ($P<0.05$).

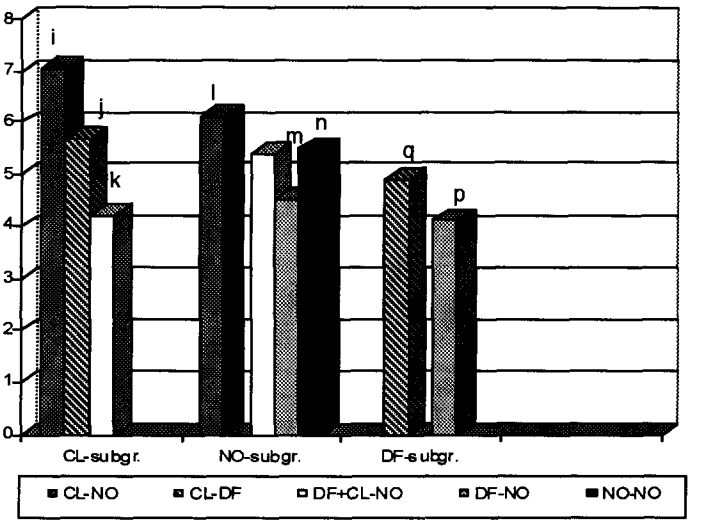
Moreover, in a similar manner as to that of oocyte yield, the pair's cleavage rate for the CL-DF pairs was higher (P<0.01) than that of the DF+CL-NO pair in these CL-bearing ovaries. There were also significant differences among the pairs of ovaries composing the NO-subgroup. The cleavage rate from the CL-NO pairs was higher (P<0.01) compared with the NO-DF pairs (6.1 ± 1.7 vs 4.5 ± 1.5, respectively) while that of NO-NO pairs differed (P<0.05) from that of NO-DF pairs (5.5 ± 1.5 vs 4.5 ± 1.5, respectively). In the DF-subgroup, the cleavage rate obtained for the DF-CL pairs was superior (P<0.01) to that of DF-NO pairs (4.9 ± 1.6 vs 4.1 ± 1.1, respectively). Although not tested, the tendency observed in these results shows that there is a correlative relationship between oocyte yield and cleavage rate after IVF. Not surprisingly, the cleavage rate demonstrated by the 5 ovarian morphological classes in this study followed the same pattern observed in the oocyte yield above.



a-b; a-c; b-c; d-e; e-f (P<0.01) and g-h (P<0.05)

Figure 2. Comparisons of oocyte yields among the subgroups.

Regarding the number of fertilized oocytes per ovary which succeeded in developing upto the blastocyst stage (Figure 4), starting with the CL-subgroup, the pairs of CL-NO resulted in a higher (P<0.01) blastocyst rate than either CL-DF or DF+CL-NO pairs of ovaries (46%, 3.3 ± 1.0 vs 35%, 2.0 ± 1.0 and 33%, 1.4 ± 0.9, respectively). Moreover, the latter pair's cleavage rate was also inferior (P<0.05) to that of CL-DF pairs. When the same indice was analyzed among the components of the NO-subgroup, the NO-CL pair yielded higher (P<0.01) rates of blastocysts than either NO-DF+CL, NO-DF, or NO-NO pairs (43%, 2.6 ± 1.1 vs 27%, 1.6± 0.7 and 33%, 1.5 ± 0.7, respectively). However, there was no significant difference observed among the DF-subgroup members. Again, the overall blastocyst rate from the 5 ovarian morphological classes in a decreasing order was: NO-CL, NO-NO, CL-DF, DF+CL-NO and DF-NO pairs.



i-j, i-k, j-k, l-m, p-q ($P<0.01$) and m-n ($P<0.05$).

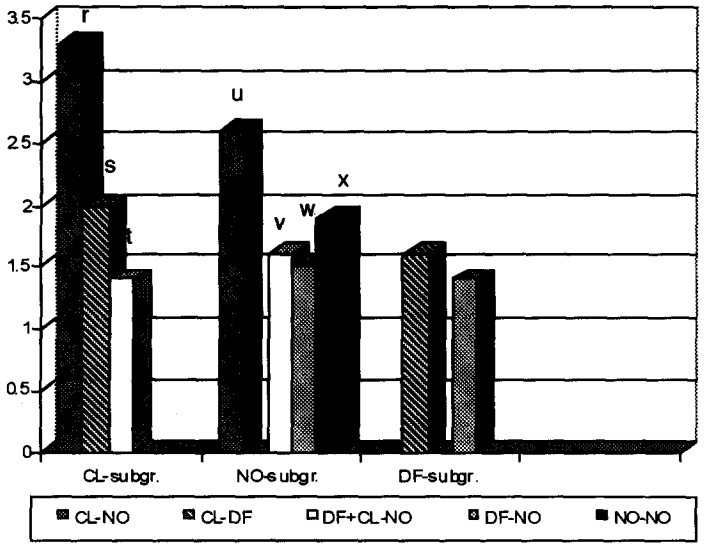
Figure 3. Cleavage rates among the 3 subgroups.

DISCUSSION

Although some attempts have been made to collect ovaries from individual cows/heifers (7, 18) for the production of IVF blastocysts, morphological classification of these ovaries prior to being aspirated and subsequent oocyte quality for use in vitro remain to be elucidated. In this study we tried to classify the ovaries (on an individual basis) according to their morphological aspects, and to see whether such characteristics have an influence on both oocyte recovery and on their subsequent quality to cleavage and blastocyst development in vitro.

The results obtained in our present study show that oocyte yield, cleavage rate and blastocyst rate are a function of ovarian morphological status, which in turn is dependent on the stage of estrous cycle of the cow. Starting with the yield of oocytes per ovary, our results show that there is wide variability among ovarian morphological classes formed, as has been previously reported (9, 18) for the in vitro developmental capacity of the oocytes collected from individual cows. Indeed, this variation in morphological ovarian classes may be due to differences between individual cows (genetic, age, and herd management) and to the status of their corresponding reproductive cycles. We observed that the NO-CL pair ovaries yielded the highest number of oocytes per ovary, followed by that of NO-NO, DF-CL and DF+CL-NO pairs. The lowest oocyte yield came from DF-NO pairs. By considering the first and last pairs of these classes based on oocyte yield, fewer oocytes were obtained from the DF-NO pair than the NO-NO pair of ovaries due to the atresia of small follicles in relation to the presence of the dominant follicle. Similar observations have been documented (22, 24, 25). In the cited studies, the development of a dominant follicle was thought to be closely associated with the regression of subordinate follicles, and new growth of the small follicles occurred only once the large dominant follicle had ceased to grow. Similar negative

effects of the presence of dominant follicle on the superovulatory response in donor animals have also been reported (4, 6, 8, 10, 11).



r-s, r-t, u-v, u-w, u-x ($P<0.01$), s-t ($P<0.05$).

Figure 4. Blastocyst rate among the subgroups.

On the other hand, the presence of corpus luteum in the pair of ovaries seems 1) to indicate that the cow is apparently cyclic (as in the NO-CL pairsin our study), and 2) to counteract the negative effects caused by the presence of a dominant follicle (as in the DF-CL; DF+CL-NO pairs), thus improving oocyte recovery rates. Although the precise endocrine control of the patterns of follicular recruitment, selection, growth and regression, particularly during the early and mid-luteal phase of the estrous cycle are not fully understood, it is thought that the secretion of high levels of estradiol, progesterone and inhibin by the corpus luteum results in a prolonged suppression of FSH and consequently, the inhibition of the development of large follicles (1, 26, 27). The reason for the superiority of the ovary pairs containing CLs over those of the NO pair ovaries are not clear. However, it may be that CL-bearing ovaries, as described above, indicate active functionality, whereas the NO pair ovaries represent the inactive phase of the corresponding ovaries. Nevertheless, further studies are needed to clarify this phenomenon.

Our results also support the concept that the intraovarian environment to which oocytes are exposed is a major cause of the variability in developmental competence of the oocytes (5, 19), since the same pattern as that of oocyte yield (per morphological class) was followed through cleavage to the blastocyst stages. These results differ from those obtained by Smith et al., (25) with regard to the developmental competence of the oocytes obtained in the presence or absence of a dominant follicle. The difference in findings between the 2 studies may be due to differences in the number of cows, breed, seasonal effects, culture media, and the technical personnel who performed the fertilization/maturation procedures. Nevertheless, although the morphological classification of the ovaries prior to follicular aspiration either from a live cow (by using ultrasound-guided equipment) or from abattoir sources could be used as an indicator of the developmental ability of the oocytes, some

individual cow variations (9, 18) will occur, probably due to genetic and herd management factors.

In summary, the present study shows that the morphological classification of the ovaries prior to follicle aspiration could be used as an indicator of developmental competence of the collected oocytes and that the presence of a dominant follicle in either one or both ovaries of a pair has a negative effect on the IVF-produced bovine embryos.

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