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Efficacy of a single measurement of plasma anti-Müllerian hormone concentration for ovum pick-up donor selection of Japanese Black heifers in herd breeding programs

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Abstract. In this study, we evaluated the efficiency of a single measurement of plasma anti-Müllerian hormone (AMH) concentration in heifers in determining the number of oocytes recoverable by ovum pick-up (OPU), and compared AMH concentrations among sister heifers from the same parents. For this, blood samples from 50 embryo-transfer-derived female Japanese Black (JB) heifers (mean: 8.7 age in months) were collected and plasma AMH concentration was measured. At 13–15 months of age, both the number of follicles (2–9 mm) and the number of collected oocytes after OPU were counted and compared. Results indicated that the heifers with the highest AMH concentration had the highest number of follicles in their ovaries and gave the highest number of collected oocytes with OPU, thereby indicating that a single measurement of plasma AMH concentration is informative for the selection of OPU-donor heifers in herd breeding programs. The practice of performing a single AMH measurement may accelerate the intensive breeding of JB herds.
Key words: Anti-Müllerian hormone, Cattle intensive breeding, Japanese Black cattle, Ovum pick-up

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The profitability of the dairy and beef industries is highly correlated with meat and milk yield, genetic selection, and reproductive efficiency. Therefore, reproductive technologies such as *in vivo* or *in vitro* embryo production have been applied worldwide to rapidly enhance the genetics of dairy and beef cattle through the female lineage [1]. The scale-up of large-scale farming and intensive production is accelerating for many breeds including the production of Japanese Black (JB) cattle in Japan. Successful breeding cattle are increasingly important not only for traditional breeding programs but also for embryo production as excellent donors for improving breeding ability. In general, 8−10-month-old JB heifers in Japan are either sold for meat or held as egg donors for breeding. The criteria used for selecting donor heifers depends on growth rate, pedigree, market conditions, temper, and meat quality based on information from their siblings. However, considerable individual variability in the responsiveness to superovulation treatment or in the number of collected oocytes after ovum pick-up (OPU) have been reported [2–4]. Although recent reports have indicated that a single ultrasound examination of the ovaries performed at pre-pubertal ages to count antral follicles can be used as a predictor of the number of oocytes that can be collected [5, 6], it is difficult to conduct rectal palpation on JB heifers due to their body size at 8−9 months old. Therefore, development of a prognostic method for determining the intrinsic capacity of a potential donor heifer to produce an expected number of oocytes or embryos is necessary.

Anti-Müllerian hormone (AMH) is a glycoprotein belonging to the transforming growth factor beta family and is secreted by ovarian granulosa cells mainly from pre-antral and early antral follicles in female [7, 8]. Recent studies have indicated that the AMH concentration in cattle is characteristic of an individual cow and that it can be considered to be a reliable endocrine marker of the number of ovulation events and embryos produced in response to superovulation or through Ovum Pick Up/*In vitro* Embryo Production (OPU-IVP) [5, 9–11]. Regarding JB cattle, a recent study of JB heifers revealed that circulating AMH concentration in heifers aged 10 or 11 months correlated with the number of embryos collected after superovulation at 13−18 months old [12]. Thus, it was concluded that the evaluation of AMH concentration during the early stage is valuable for the selection of candidate embryo donors in JB cattle [12]. Based on these findings, we speculated that a single measurement of AMH concentration in JB heifers aged approximately 9−10 months may be useful for predicting the breeding potential of OPU-donor heifers undergoing routine OPU treatment 3−4 months after the AMH measurement.

The number of blastocysts produced by OPU-IVP is dependent upon the number of recovered oocytes [13, 14], and the selection of donor cows for an OPU-IVP program seems to be the best approach to optimize the number of highly competent oocytes and embryos produced [14]. Thus, we carried out the present study to progress the efficiency of genetic improvement and embryo production by routine OPU trial. We selected heifers from a commercial farm based on measurements of AMH concentration. All heifers were derived

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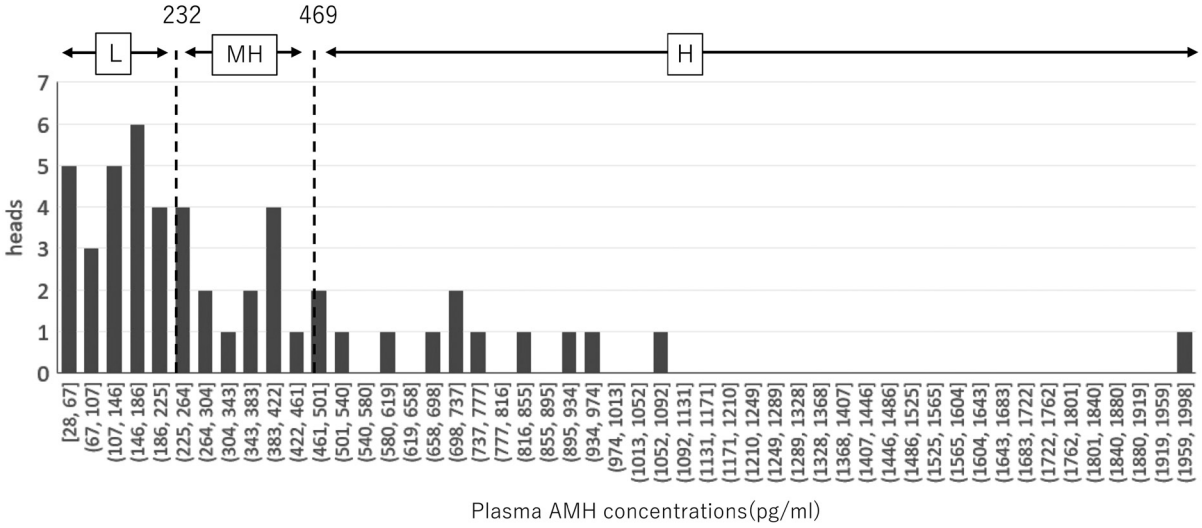

Fig. 1. Frequency distribution of plasma anti-Müllerian hormone (AMH) concentration (n = 50). Group H: highest 25% of heifers; Group MH: next 25% of heifers; Group L: lowest 50% of heifers.

from multiple ovulation and embryo transfer (MOET) production.

The objective of the present study was to evaluate the relationship between a single plasma AMH concentration measurement in heifers aged approximately 7–10 months and the number of oocytes recovered after OPU treatment at 13–15 months of age. Additionally, AMH concentration was compared among sister heifers from the same parents derived from MOET.

Plasma anti-Müllerian hormone concentrations, classification, and heifer selection

Plasma AMH concentrations of 50 JB heifers ranged from 28−1,998 pg/ml, and the mean AMH concentration (± SD) was 363.5 ± 353.3 pg/ml. The median and 75th percentile values of AMH concentration were 232 and 469 pg/ml, respectively. The histogram of AMH concentration of each heifer and the range of AMH concentration for each group are shown in Fig. 1. Based on the criteria, the 50 heifers were classified into H (n = 12), MH (n = 13), and L (n = 25) groups, and then selected as donors for OPU as group H (n = 10: age in months, 8.8 ± 1.6), group MH (n = 6: age in months, 8.6 ± 0.8), and group L (n = 9: age in months, 8.6 ± 1.1). There were no significant differences among the ages of each group.

Number of follicles by ultrasound monitoring and collected oocytes after ovum pick-up

The age of the examined heifers during the first OPU carried out on groups H, MH, L, and C were 412.5 ± 18.2, 418.2 ± 19.7, 402.1 ± 11.4, and 412.1 ± 49.5, and there were no significant differences among the groups. The number of follicles determined via ultrasound examinations during OPU are shown in Fig. 2. Although the number of follicles 2–9 mm in diameter between the first and third OPU trials were not significantly different within each group, the mean numbers of follicles from group H were significantly higher ($P < 0.05$) than the other three groups, not only during each trial of OPU but also in terms of the average of three OPU trials.

The number of collected oocytes after OPU are shown in Fig. 3. The mean numbers of collected oocytes between the first and third OPU trials were not significantly different for each group of heifers. The mean numbers of oocytes from group H was significantly higher ($P < 0.05$) than that of the other three groups, not only at each trial of OPU but also the average of three OPU trials. Plasma AMH concentrations was positively correlated with the total number of follicles ($R^2 = 0.3832$, $P < 0.05$) and oocytes ($R^2 = 0.3237$, $P < 0.05$) as an average of three OPU trials, as shown in Fig. 4.

Comparison of anti-Müllerian hormone concentrations among embryo-transfer-derived sister heifers

AMH concentration of sister heifers derived from the same parents by MOET is shown in Table 1. In the present study, two to five MOET-derived sister heifers were born from 10 donor cows. Although some sister heifers from the same donor parents showed similar AMH concentration (such as three heifers from donor A: 226, 271, and 373 pg/ml; or two heifers from donor I: 436 and 516 pg/ml), extremely different AMH concentration was observed among other sister heifers (such as five heifers from donor J: 35, 161, 246, 271, and 720 pg/ml; or three heifers from donor F: 157, 921, and 1998 pg/ml).

Recently, Hirayama *et al.* [12] reported that the evaluation of AMH concentration during the early stages is valuable for selection of candidate embryo donors in JB cattle. Therefore, in the present study, we applied the measurement to select JB donor heifers for OPU-IVP to a commercial JB herd. In the OPU-IVP system, many factors during the *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture processes affect the blastocyst rates obtained from OPU-derived oocytes. Additionally, previous reports indicated that the number of blastocysts produced by OPU-IVP is dependent on the number of recovered oocytes [13, 14]. Therefore, in the present study, we firstly focused on the relationship between AMH concentration and both the number of follicles and recovered oocytes. Our results

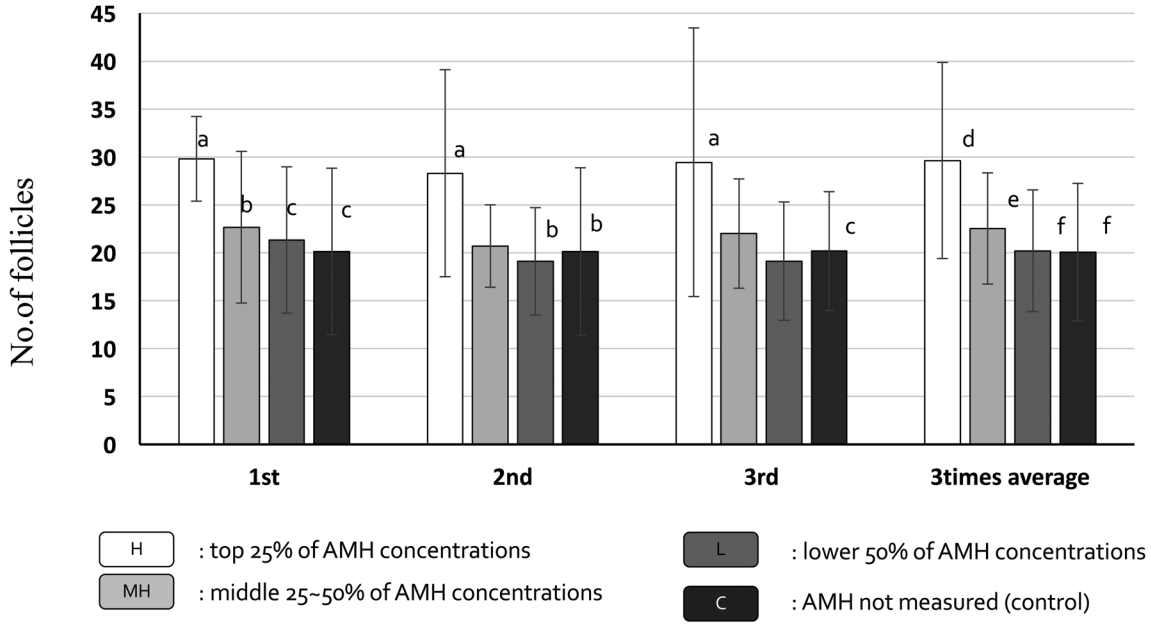

Fig. 2. Number of follicles observed by ultrasound at first and third ovum pick-up (OPU) trial, and average of three OPU trials. a–b, d–e: significant differences ($P < 0.05$), a–c, d–f: significant differences ($P < 0.01$)

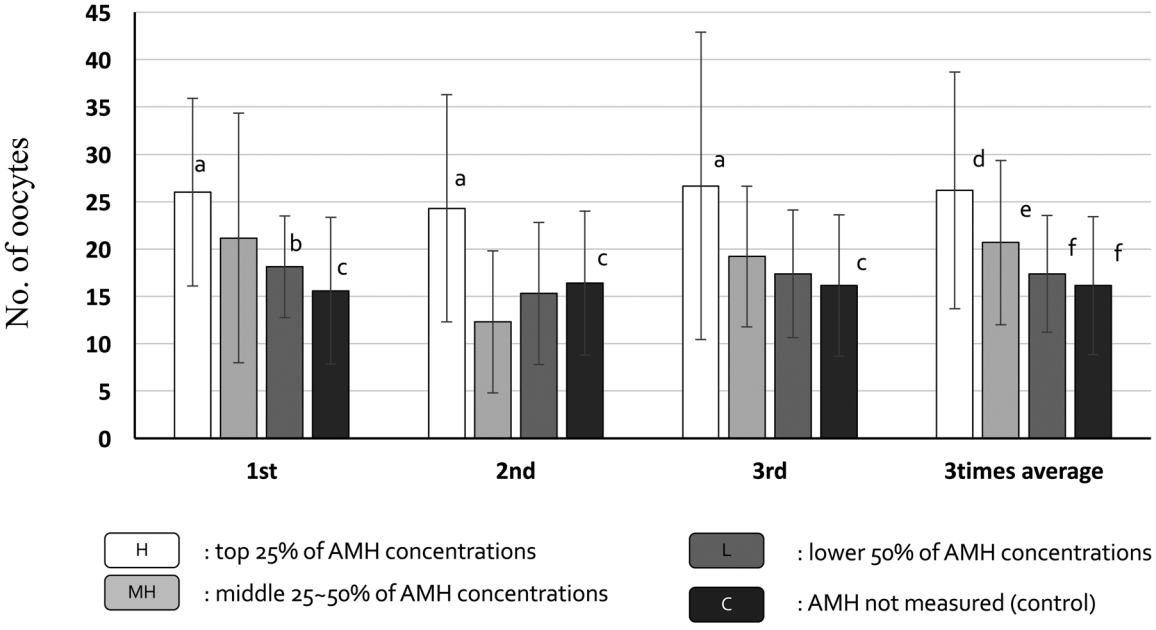

Fig. 3. Number of collected oocytes at first and third ovum pick-up (OPU) trial, and average of three OPU trials. a–b: significant differences ($P < 0.05$), a–c, d–f: significant differences ($P < 0.01$), d–e: tend to significant difference ($0.05 < P < 0.1$)

clearly indicated that the heifers with higher AMH concentration (> 469 pg/ml) measured at 7–10 months of age had the highest numbers of follicles in their ovaries and gave the highest number of oocytes during OPU carried out at 13–15 months of age.

It has been suggested that AMH concentration is a reliable phenotypic marker, not only for size of the ovarian reserve, ovarian function, and response to superovulation, but also for fertility and herd longevity of cattle [5, 17, 18]. Recently, in dairy cattle, it was suggested that AMH might be useful to indirectly improve predictions of the genetic merit of some reproductive traits and that there

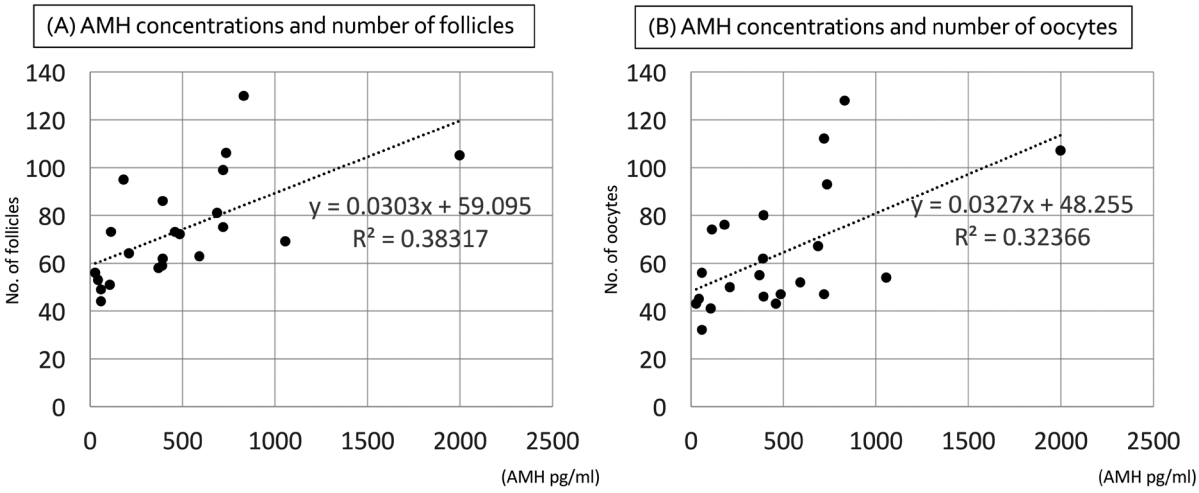

Fig. 4. (A) Correlation between total numbers of follicles and plasma anti-Müllerian hormone (AMH) concentration (n = 25, P < 0.01); (B) Correlation between total numbers of collected oocytes and plasma AMH concentration (n = 25, P < 0.01).

Table 1. Anti-Müllerian hormone (AMH) concentrations of sister heifer derived from the same parents with multiple ovulation and embryo transfer from 10 donor cows

Donor (n = 10)	A	A	B	C	D	E	F	G	G	H	I	I	J
Father (n = 6)	a	b	c	d	b	e	f	a	f	a	a	e	f
No. of sister heifers	2	3	4	3	2	2	3	2	4	2	2	2	5
AMH concentration (pg/ml) of each sister heifers	133	226	165	124	185	193	157	201	184	71	61	436	35
	593	271	226	335	395	1057	921	689	401	395	197	516	161
		373	833	462			1998		722				246
			973						738				271
													720

may be a possible relationship between AMH concentration and superovulatory traits in dairy cattle [18]. Additionally, regarding the application of AMH concentration for OPU trials, previous reports also considered AMH to be a reliable endocrine marker of the number of ovulations and embryos produced in response to superovulation or through OPU-IVP [5, 9–11]. In the present study, measurement of AMH from each heifer was once carried out when rectal palpation of the heifer was difficult because of their body size and at the time when owner's make decisions regarding the fate of the heifers. It has been reported that plasma AMH concentration in JB cows ranged from 0.032−1.992 ng/ml, and mean ± SD was 0.334 ± 0.318 [12]. In the present study, although the age of the heifer when blood was sampled was different to that of the heifers tested in the previous report, the AMH concentration was almost within the same range, and the average was almost similar. Therefore, although it was suggested that animal breed and age represented major factors influencing AMH concentration in cattle [14, 19], our results suggest a general AMH concentration for JB cattle.

In the present study, we used the median and 75th percentile values of AMH concentration for classification of 50 heifers according to the previous report which was evaluated the superovulatory response of JB cattle [12]. Although we used the classification methods for evaluating both the number of follicles and collected oocytes after OPU trials of JB heifers, our results clearly indicated that were significant differences for both parameters among each classified group. Our results may indicate that the classification method for heifers by a single measurement of AMH concentration is useful for selecting OPU-donor heifers for herd breeding programs.

Rico *et al.* [20] reported that the measurements of plasma AMH concentration before each repetition of the OPU protocol were highly repeatable over a one-year period, similar to counts of the number of follicles that developed after treatment and were available for follicular puncture. In agree with the previous report, our results also reconfirm a strong within-animal repeatability in the numbers of both follicles and oocytes in 13–15 months old JB heifers, for at least three repeats of the OPU trials.

In the present study, among the 50 examined heifers, two to five MOET-derived sister heifers were produced from 10 donor cows using the frozen semen of six bulls. Interestingly, although some sister heifers from the same donor parents had similar AMH concentrations, extremely different AMH concentrations were observed among other sister heifers under the same nutritional conditions. Studies have indicated that AMH concentration is highly variable among individuals, and that the factors affecting ovarian reserves (indirectly

affecting circulating AMH concentration) may be maternal nutrition, diseases and the presence of endocrine disrupters during pregnancy [5, 19, 21]. Therefore, our results regarding the differences observed among heifers within each family may strongly support these previous reports. Nevertheless, we propose the exclusion of dietary feed as a factor affecting ovarian reserves and instead add as factors genetic differences between sisters, environmental differences related to the nutritional conditions, and low appetite of the recipient cows/heifers when affected by disease. Moreover, our results suggest that the environmental management of recipient cows/heifers, including nutritional control and disease prevention, is important for improving the breeding ability of the herd.

In conclusion, the present study revealed that a single measurement of plasma AMH concentration at 7–10 months old could be an efficient method for selecting donor heifers for OPU or superovulation. It would therefore accelerate the intensive breeding of JB herds. Additionally, our results may reconfirm that AMH concentrations in JB heifers is highly variable among individuals, and that the factors that indirectly affect AMH concentration may be maternal nutrition, disease, and the presence of endocrine disrupters during pregnancy.

Methods

The experiments were conducted according to the regulations concerning the protection of experimental animals and the guidelines of Yamaguchi University, Japan (No.40, 1995, approval date 27 March 2017).

Animals and blood sampling

The experiment was carried out with 50 JB heifers (mean ± SD, 8.7 ± 0.7 age in months; range: 7.3–10.2 months-old) that were held at a commercial farm in Hokkaido, Japan, from October 2016 to August 2017. They were all born at the same farm derived from a MOET program, and their mothers, recipient F1 heifers, were held in the same environment and provided the same feed (roughage: 10.5 kg; dent-corn silage: 1 kg; concentrate 3.3 kg; and calculated required minerals) during their pregnancy. Venous blood samples for AMH measurement were collected once from heifers immediately prior to the owner's decision regarding whether the heifer will become an egg donor or be sent to the market. After collection in silicone-coated tubes, the blood samples were centrifuged at 3000 × *g* for 10 min to recover the plasma, which was stored at −20°C until the AMH assays were carried out.

Measurement of plasma anti-Müllerian hormone concentration and classification of the heifers

Plasma AMH concentration was measured within 7 days of collection with a Bovine AMH ELISA kit (AnshLabs, TX, USA) according to the previous report [15]. Undiluted plasma (50 µl) was used for the assay, and the assay had a limited detection of 11 pg/ml and a coefficient of variation of 2.9 according to the manufacturer's instructions. Based on AMH concentration, animals were divided into three groups as follows: group H contained those with the highest 25%, group MH contained those with the next 25%, and group L contained those with the lowest 50% of AMH concentration. Then, 10 out of 12 of group H, six out of 13 of group MH, and nine out of 25 of group L animals were selected to be held at the farm as donors for OPU. The other 25 animals were sold on the market. Although the AMH concentration of the heifers of group L was low, they were selected under the conventional evaluation criteria to be used as donors, specifically based on growth, pedigree, and temper. In the present study, 84 heifers without measured plasma AMH concentration were included in the control (group C) for the OPU trials.

Hormone and ovum pick-up treatments and evaluation of cumulus-oocyte complexes

At 13–15 months of age, OPU was carried out on the 25 selected heifers three times every 2 weeks by three skilled technicians. Before OPU treatment, the synchronization of follicular waves of each heifer was performed with controlled internal drug release device (CIDR, Pfizer Japan, Tokyo, Japan) insertion and GnRH (100 µg/heifer, Conceral 100, InterVet, Tokyo, Japan) administration concurrently to induce ovulation of all large follicles present in the ovary at Day 1. Three days after insertion, administration of FSH (10 AU/heifer, Antorin R-10, Kyoritsu Seiyaku, Tokyo, Japan) administered twice daily in decreasing doses over 2 days (4 days: AM, PM, 3 AU; 5 days: AM, PM, 2 AU). At 6 days, OPU was carried out 12 h after the last FSH administration after removal of the CIDR.

The number of follicles were determined via ultrasound examination immediately prior to oocyte aspiration, and the number of follicles 2–9 mm in diameter were counted. Follicular aspiration was performed using an ultrasound-guide transvaginal aspiration system (HS-2200V, FHK, Tokyo, Japan) equipped with a disposal 18-G single-lumen sterile needle using 70−80 mmHg of suction pressure. The follicles were aspirated into a tube containing a Ringer's lactate solution (ZENOAQ; Fukushima, Japan) supplemented with heparin (10 IU/ml; Ajinomoto, Tokyo, Japan) and 0.5% fetal bovine serum. The cumulus-oocyte complexes (COCs) were evaluated immediately following aspiration as per the method of de Loos *et al*. [16]. COCs with several layers of cumulus cells and homogeneous oocyte cytoplasm were classified into grade 1, while those with one to three layers of cumulus cells, completely denuded oocytes, expanded cumulus cells, and degenerated oocytes were classified into grades 2, 3, 4, and 5, respectively. After evaluation, only the numbers of grades 1 and 2 COCs were recorded as collected oocytes and the results were compared within and among the groups.

Statistical analysis

All results are presented as means ± SD. Data were analyzed using Student's *t*-test or one-way ANOVA followed by post-hoc test for comparisons between means. For all analyses, $P < 0.05$ was considered as significant.

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