

—Technology Report—

Efficacy of a single blood anti-Müllerian hormone (AMH) concentration measurement for the selection of Japanese Black heifer embryo donors in herd breeding programs

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Abstract. We evaluated the relationship between plasma anti-Müllerian hormone (AMH) concentrations in Japanese Black (JB) heifers at 7–10 months of age and the number of embryos recovered after superovulation treatment in selected ovum pick-up donors, concomitantly with changes in their AMH concentrations before and after parturition. Plasma AMH concentrations in heifers were positively correlated with the total number of follicles ($r = 0.647$, $P < 0.01$) and embryos ($r = 0.681$, $P < 0.01$) recovered from the animals postpartum, when selected as donor cows, but did not correlate with the total number of transferable embryos. No difference was observed between the plasma AMH concentration at the heifer period and the postpartum period. Additionally, serum AMH concentrations of heifers weakly correlated with the number of follicles and embryos recovered by virgin flush after superovulation treatment at 13–15 months of age. Therefore, a single blood AMH concentration measurement may accelerate intensive JB cattle breeding.

Key words: Anti-Müllerian hormone, Cattle intensive breeding, Japanese Black cattle, Ovum pick-up
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The expansion of large-scale farming for intensive cattle production is accelerating for many animal breeds, including the Japanese Black (JB) cows in Japan. Successful cattle breeding is considered increasingly important, for both traditional breeding programs and the production of excellent embryo donors to improve breeding ability. The production capacity of an embryo donor is a reproducible and heritable trait in multiple ovulation embryo transfer (MOET) and in ovum pick-up and in-vitro production (OPU-IVP) protocols [1, 2]. Therefore, predicting the response of each heifer or cow to gonadotropin treatment could allow identification of low-responsive animals at an earlier stage, resulting in their exclusion as donors, thereby improving the results of these protocols [2].

The anti-Müllerian hormone (AMH) is a glycoprotein that is secreted by ovarian granulosa cells mainly from the pre-antral and early antral follicles of females [3, 4]. Recent studies have shown that blood AMH concentration can be a reliable endocrine marker for the number of ovulation events and embryos produced in response to superovulation or OPU-IVP [1, 5–9]. A recent study on JB heifers revealed that evaluation of AMH concentration during the early stage of life is important for the selection of candidate embryo donors [10, 11]. Under farming conditions, the application of practical methods to select JB heifers as oocyte and/or embryo donors varies depending

on the breeding strategy in each farm. Based on previous findings, we reported the practical efficacy of a single AMH measurement in JB heifers at approximately 7–10 months of age, for predicting the breeding potential of OPU-donor heifers undergoing routine OPU treatment at approximately 13 months of age [12]. Regarding OPU donors, Rico *et al.* [2] reported no significant effect of a repeated number of OPU sessions following gonadotropin treatment on the numbers of large follicles or plasma AMH concentrations over a one-year period. Moreover, Nabenishi *et al.* [11] had reported that the time course of mean plasma AMH concentrations of JB heifers undergoing the MOET program did not significantly differ from 3 months pre-artificial insemination to 3 months postpartum. However, in the case of MOET donors, repeated superovulation in JB cows (2.6–8.8 years of age) resulted in a decrease in the average plasma AMH concentration with a reduction in the number of ova and embryos [10]. The long-term change in plasma AMH concentration for selecting OPU-IVP donors, as well as its relationship with postpartum superovulation response after the first calving, is unclear, especially in JB heifers, and warrants further research. Another option for selecting donor JB heifers for the MOET strategy is a virgin flush approach. However, to the best of our knowledge, few reports [10] are available on the correlation between embryo yields by virgin flush in JB heifers and AMH concentrations.

The present study therefore aimed to evaluate the relationship between a single plasma AMH concentration measurement in JB heifers at approximately 7–10 months of age, and the number of follicles by transrectal ultrasound examination as well as the recovery of embryos postpartum by superovulation treatment conducted after the first calving in selected donors. We also investigated the relationship between serum AMH concentrations in heifers and the

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number of recovered embryos after the MOET program at 13–15 months of age, by virgin flush, to evaluate the efficacy of this breeding strategy in JB cattle herds.

Previously, we studied 25 OPU-donor heifers [12], 16 of which were selected as donor cows based on the AMH concentration measured during the heifer period for a farm-based postpartum MOET program in the present study. Heifers with low AMH concentrations (< 232 pg/ml) were excluded, so that the AMH concentration of the selected heifers ranged from 403 to 1998 pg/ml. Plasma AMH concentrations measured in heifers were positively correlated with the total numbers of follicles ($r = 0.647$, $P < 0.01$; Fig. 1A) and recovered embryos ($r = 0.681$, $P < 0.01$; Fig. 1B) from the same animals postpartum, but did not correlate with the total number of transferable embryos ($r = 0.170$, $P = 0.528$; Fig. 1C).

In the 16 animals examined, no significant difference was observed between the plasma AMH concentration measured during the heifer period (789.4 ± 402.8 pg/ml) and the postpartum period for the first MOET trial (i.e., 27–33 months of age; 746.8 ± 370.4 pg/ml). The second AMH concentration values were lower in 75% of the heifers compared with the first, and the remaining four heifers showed increased AMH concentrations after parturition. Additionally, the two AMH concentrations correlated positively ($r = 0.724$, $P < 0.01$; Fig. 2A). There was also a significant correlation in the mean number of follicles pre- and post-pregnancy ($r = 0.599$, $P = 0.014$; Fig. 2B). The change in AMH concentrations, expressed as a ratio of the concentrations pre- and post-partum and known as the AMH ratio (second AMH concentration/first AMH concentration), positively, but moderately correlated with the number of OPU sessions (2 to 9 times) conducted before pregnancy ($r = 0.520$, $P = 0.039$; Fig. 2C). This suggests that OPU sessions have no adverse effects on the antral follicle ovarian reserve.

Serum AMH concentrations measured in the 63 heifers selected for the MOET program (virgin flush) positively correlated with the total number of follicles ($r = 0.261$, $P = 0.039$; Fig. 3A) and recovered embryos ($r = 0.244$, $P = 0.055$; Fig. 3B), but did not correlate with the total number of transferable embryos ($r = 0.172$, $P = 0.177$; Fig. 3C).

Based on studies for the efficiency of AMH concentration in the selection of candidate embryo- or OPU-donor cattle [1, 5–7, 10, 11], we have previously reported that a single plasma AMH measurement at 7–10 months of age was an effective method for selecting JB donor heifers, especially for OPU purposes [12]. In the present study, we first focused on the relationship between a single plasma AMH concentration in JB heifers and the number of embryos recovered via superovulation treatment after first parturition of selected OPU-IVP donors. Our findings clearly indicated that heifers with a high AMH concentration produced a high number of follicles in their ovaries and a high number of embryos following their postpartum MOET program, although no correlation was observed with the quality of the recovered embryos. Additionally, we observed positive correlations between the first and second AMH concentrations, and the number of follicles per OPU session before pregnancy and the mean number of follicles per superovulation trial after pregnancy. Rico *et al.* [2] reported that measurement of plasma AMH before each repeat round of the OPU protocol was highly reproducible over a one-year period, similar to enumerations of the number of follicles that developed after treatment and that were available for follicular puncture. In

our previous report [12], we also confirmed a strong within-animal reproducibility of the numbers of both follicles and oocytes in JB heifers at 13–15 months of age for at least three OPU session repeats. Overall, the findings of the present study strongly corroborate those of the previous study [2], suggesting that the ovarian reproducibility of the numbers of both follicles and oocytes in each animal do not affect plasma AMH levels, at least after the first parturition, even after multiple OPU sessions.

Rico *et al.* [2] reported no significant effect of repeated OPU or superovulation treatments on follicular number or plasma AMH concentrations in Holstein donor cows. However, Hirayama *et al.* [10] reported that repeated superovulation in JB cows results in a decrease in the average plasma AMH concentration, along with a decrease in the number of ova and embryos, and is not related to their parity. This difference may be due to the difference in the levels of ovarian reserves before gonadotropin treatment [10]. On the other hand, previous examinations of the effects of repeated OPU (4–5 weeks, twice weekly) on ovarian morphology parameters in dairy heifers indicated slight alterations in endocrine profiles, as well as minor morphological changes in the ovaries [13]. Additionally, Monniaux *et al.* [14] suggested that AMH endocrine levels decrease during the days following estrus in response to pre- and peri-ovulatory FSH surges. Thus, we initially expected that minor ovarian disorders after repeated OPU sessions might be accompanied by changes in AMH concentrations. Although the AMH concentration from 75% of donor cows (i.e. 12 out of 16 animals) re-measured at the first postpartum MOET program decreased and the mean AMH concentrations at both time points from the 16 cows were not significantly different, there was a moderately positive correlation between the number of OPU sessions before pregnancy and the AMH ratio. These data indicate that more the number of OPU sessions before pregnancy, greater the increase in AMH concentration after pregnancy, suggesting no adverse effects of OPU sessions on the ovarian reserve of antral follicles. Although the age of the animals examined in the present study varied from the previous studies (4–9 years in Rico *et al.* [2], and 2.6–8.8 years in Hirayama *et al.* [10]), repeated OPU sessions with gonadotropin treatment during the heifer period did not appear to adversely affect the number of antral follicles in the ovary and the AMH concentration. Interestingly, the remaining 25% of donor cows showed increased AMH concentrations when re-measured after their first parturition. Previously, factors such as nutrition, hormones (e.g., endocrine disrupting chemicals), and diseases (e.g., granulosa-theca cell tumor and mastitis) appeared to influence the ovarian reserve, and hence, the AMH concentration of female animals [7, 15–17]. All the examined donor heifers investigated in the present study were nurtured under the same environmental and feeding conditions on the same farm, without records of clinical treatment for any disease. Therefore, the physiological mechanisms underlying these changes in AMH concentration remain unclear from the present study. Further research targeting the physiological mechanisms may contribute to improve reproductive efficacy in the cattle industry.

In the present study, we also focused on the relationship between a single serum AMH concentration in JB heifers and the number of embryos recovered via superovulation treatment. We observed a weak positive correlation between the AMH concentration and numbers of both the follicles ($r = 0.261$) and recovered embryos

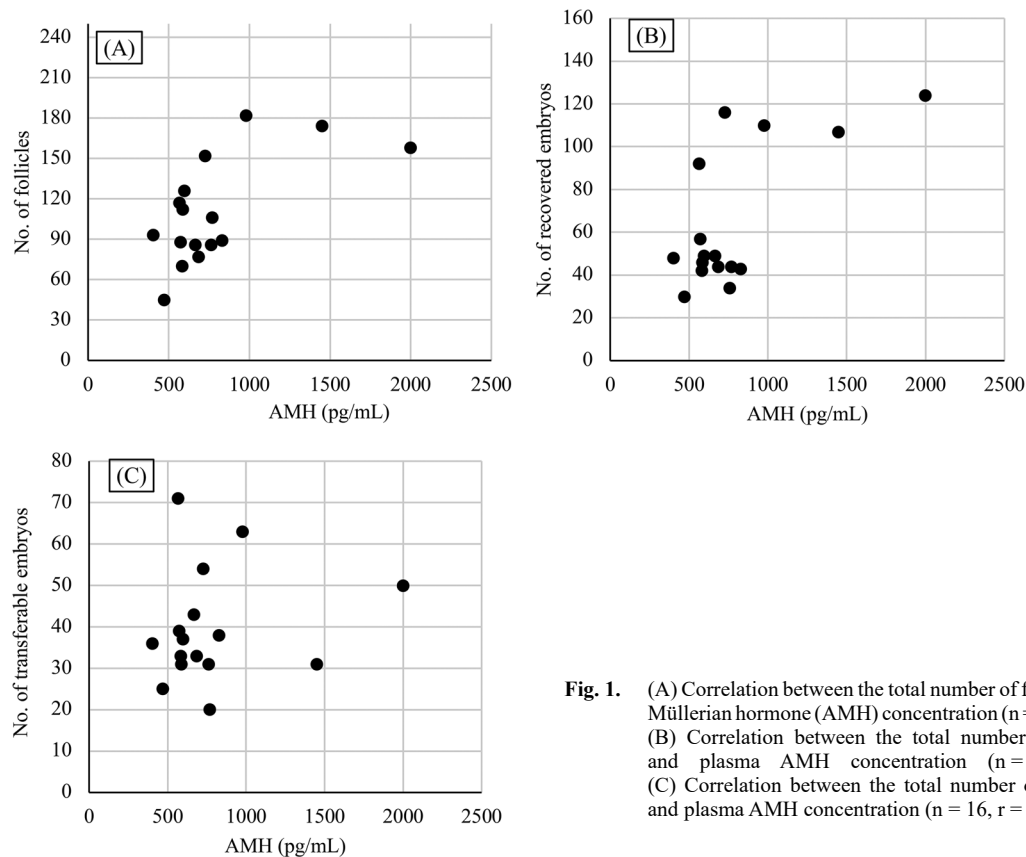


Fig. 1. (A) Correlation between the total number of follicles and plasma anti-Müllerian hormone (AMH) concentration ($n = 16$, $r = 0.647$, $P < 0.01$). (B) Correlation between the total number of embryos recovered and plasma AMH concentration ($n = 16$, $r = 0.681$, $P < 0.01$). (C) Correlation between the total number of transferable embryos and plasma AMH concentration ($n = 16$, $r = 0.170$, $P = 0.528$).

($r = 0.244$), and no correlation with the number of transferable embryos. Hirayama *et al.* [10] reported a significant positive correlation ($r = 0.641$) between plasma AMH concentration measured in 10–11-month-old heifers and number of embryos after conducting superovulation treatment at 13–18 months of age. In the present study, a greater positive correlation between AMH concentration and numbers of both the follicles ($r = 0.647$) and recovered embryos ($r = 0.681$) was obtained from 16 OPU-donor heifers. The age at which virgin flush was initiated in the heifers may affect the differences observed between the Hirayama *et al.* [10] and the current study. Therefore, when selecting candidate heifers for virgin flush using the AMH concentration as an index in the herd, the timings of AMH concentration measurement, and the superovulation treatment might be critical determinants. Furthermore, in the present study, heifers with low AMH concentration were excluded from the virgin flush, which may have caused the variation from previous study. Even so, our results may indicate the additional possibility of virgin flush based on the one-time measurement of serum AMH concentration during the heifer period as a potential strategy in JB herd breeding programs. Recently, blood AMH concentration has been suggested to be a reliable phenotypic marker, not only for the size of ovarian reserve, function, and response to superovulation, but also for cattle fertility and herd longevity [7, 18, 19]. Therefore, further studies focusing on the fertility of these selected donor heifers may be crucial in ensuring the longevity of JB cattle.

In conclusion, the results obtained clearly indicate that heifers with the highest AMH concentrations, measured at 7–10 months of age, produce the most embryos after superovulation treatments during the first postpartum period. Further studies are required to investigate the lack of differences in the number of transferable embryos observed after superovulation treatments. Overall, a single measurement of blood AMH concentration may accelerate intensive breeding of JB cattle herds.

Methods

Experiments were conducted according to the regulations for the protection of experimental animals and guidelines stipulated by Yamaguchi University, Japan (No. 40, 1995; approved on March 27, 2017).

Animals targeted in the present study

Details on the animals and collected plasma samples used in the present study are as described in our previous study [12]. Briefly, female JB heifers from a single farm subject to the same feeding regime and rearing conditions were used in this study. Blood samples were obtained from 50 heifers aged 7.3–10.2 months (average age, 8.7 months) before the owner deciding whether it would become an egg donor. Plasma AMH concentrations were measured using a commercially available ELISA kit (AnshLabs, Webster, TX, USA)

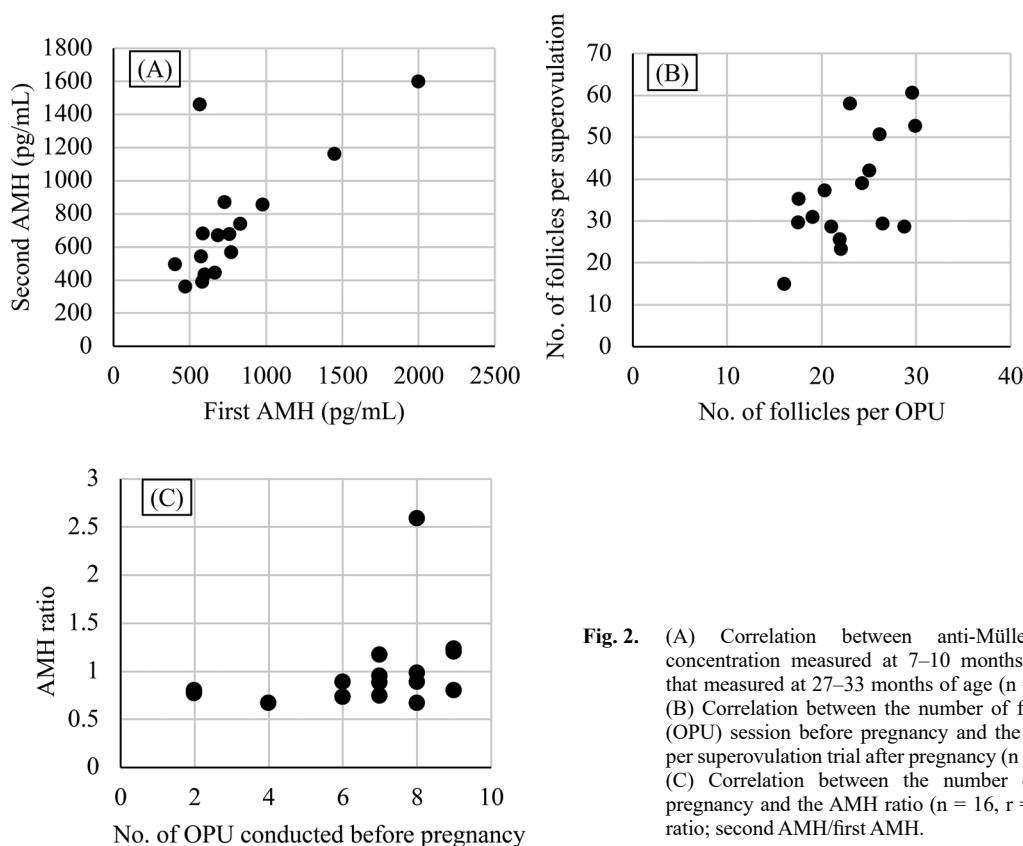


Fig. 2. (A) Correlation between anti-Müllerian hormone (AMH) concentration measured at 7–10 months of age (first AMH) and that measured at 27–33 months of age ($n = 16$, $r = 0.724$, $P < 0.01$). (B) Correlation between the number of follicles per ovum pick-up (OPU) session before pregnancy and the mean number of follicles per superovulation trial after pregnancy ($n = 16$, $r = 0.599$, $P = 0.014$). (C) Correlation between the number of OPU sessions before pregnancy and the AMH ratio ($n = 16$, $r = 0.520$, $P = 0.039$). AMH ratio; second AMH/first AMH.

[20]. Based on the 25th, 50th (median), and 75th percentiles of mean AMH concentrations, 25 heifers were selected as OPU donors and divided into three groups (group H: high AMH concentration, $n = 10$; group MH: intermediate concentration, $n = 6$; and group L: low concentration, $n = 9$) [12]. Among these 25 OPU-donor heifers, 16 from groups H and MH were further subjected to the MOET program. The OPU treatments conducted after the age of 13 months have been described previously [12]. Briefly, synchronization of follicular waves in each heifer was achieved using an internal drug release insert (CIDR; Pfizer Japan, Tokyo, Japan) and administration of GnRH (100 μg /heifer, Conceral 100, MSD Animal Health, Tokyo, Japan) concurrently at day 1. FSH (10 armour unit (AU)/heifer, Antorin R-10; Kyoritsu Seiyaku, Tokyo, Japan) was administered twice daily, in decreasing doses over two days (i.e., 3 AU at day 4 and 2 AU at day 5). On day 6, OPU was carried out 12 h after the last FSH administration and CIDR removal. The total number of follicles was determined via ultrasound examination immediately prior to oocyte aspiration before the first pregnancy.

Superovulation treatment and plasma AMH concentration measurement of selected postpartum OPU-donor heifers

Of the 16 donor cows, one cow underwent a caesarean section during parturition, and the remaining 15 had normal calving. Mother-infant separation was performed immediately after calving, and the donor cows were not milked. The 16 donor cows exhibited normal

postpartum uterine recovery and had no history of diseases. Embryo collection after superovulation treatment was performed at two-month intervals from the age of approximately 27–33 months (mean age: 29.9 ± 2.1 months). The OPU donors that had a normal first postpartum estrus cycle received a superovulation treatment from 9 to 14 days after the first day of the second postpartum estrus. Synchronization of follicular waves in each cow was achieved using a CIDR on the morning of day 0, and estradiol (2 mg, Ova Hormone, ASUKA Animal Health, Tokyo) was administered on day 6. FSH was administered (20 AU/heifer, Antorin R-10, Kyoritsu Seiyaku) twice daily, with the dose decreasing over three days (i.e., 5 AU on day 10, 3 AU on day 11, and 2 AU on day 12, both in the morning and evening). $\text{PGF}_{2\alpha}$ (2 ml, Estrumate, MSD Animal Health) was injected in the morning and evening on the third treatment day, and subsequently CIDR was removed. Donor cows were artificially inseminated after GnRH administration (100 μg /heifer) on day 14, and embryos were non-surgically recovered by two highly skilled technicians on day 7 after insemination. The number of follicles (2–9 mm in diameter) observed by ultrasound examination, immediately prior to embryo flushing, and the number of embryos collected after flushing were counted. Embryos were classified according to the criteria determined by the International Embryo Technology Society, and embryos labelled with codes 1–3 were defined as transferable. Plasma samples from all the 16 donor cows were collected at the time of postpartum first embryo recovery and stored as described previously. AMH

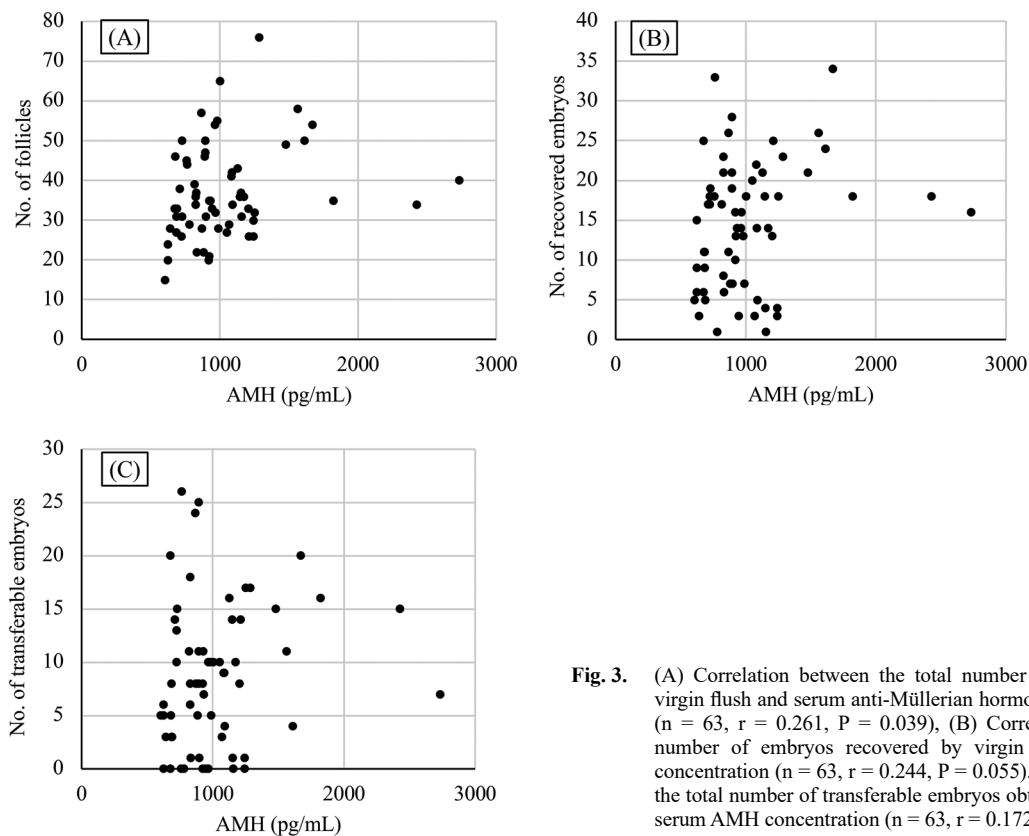


Fig. 3. (A) Correlation between the total number of follicles obtained by virgin flush and serum anti-Müllerian hormone (AMH) concentration ($n = 63$, $r = 0.261$, $P = 0.039$), (B) Correlation between the total number of embryos recovered by virgin flush and serum AMH concentration ($n = 63$, $r = 0.244$, $P = 0.055$). (C) Correlation between the total number of transferable embryos obtained by virgin flush and serum AMH concentration ($n = 63$, $r = 0.172$, $P = 0.177$).

concentrations were subsequently measured within the same ELISA assay as mentioned above. The initial (at 7–10-months of age) and postpartum AMH concentrations from the same donor cows were compared as an AMH ratio (second AMH concentration/first AMH concentration) to evaluate the effects of the number of OPU sessions as well as the gestation, parturition, and superovulation treatment.

Superovulation treatment for “virgin flush” of donor heifers selected based on the serum AMH concentration

Based on the AMH ELISA kit protocol used in the present study, a very high correlation ($r = 0.99$; $P < 0.0001$) was obtained between the plasma and serum samples. Therefore, serum sample was used as the test sample for performing virgin flush. The 25th, 50th, and 75th percentiles of the accumulated serum AMH concentrations from 187 heifers in the examined cattle herd were determined at 7–10 months of age, and 93 donor heifers were selected from the 50th percentile (AMH concentration: > 461 pg/ml). In the present study, 63 heifers among the selected 93 donor heifers were then subjected to superovulation treatment by virgin flush at 13–15 months of age as described above.

Statistical analysis

All data were analyzed using Ekuseru-Toukei 2012 for Windows, version 1.11 (Social Survey Research Information, Tokyo, Japan) and presented as mean \pm SD. Plasma AMH concentration measured

at heifer and the postpartum period for the first MOET trial were evaluated using a paired t-test. Association of AMH concentration with the number of follicles, recovered embryos and transferable embryos, association between first and second AMH concentrations, association between the number of follicles per OPU session and follicles per superovulation were evaluated using Pearson’s correlation coefficient. Association of the number of OPU sessions conducted before pregnancy with AMH ratio was evaluated using Spearman’s correlation coefficient by rank. A value of $P < 0.05$ was considered significant.

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