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Serum concentrations of anti-Müllerian hormone modulate ovarian response to different doses of follicle-stimulating hormone in Japanese Black donors

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Abstract. Anti-Müllerian hormone (AMH) is a marker for predicting embryo production in cows subjected to superovulation; however, it remains to be established as a reliable predictor of reproductive performance. We hypothesized that the serum AMH concentration of donors affects the ovarian response to follicle-stimulating hormone (FSH) treatment during superovulation. Herein, we retrospectively investigated the association between different FSH doses and AMH concentrations in donor Japanese Black cows in a superovulation program and analyzed the number of total and transferable embryos recovered. The number of transferable embryos recovered from donors with high AMH levels was significantly higher than that recovered from donors with low AMH levels. Additionally, it increased further with a reduction in the FSH dose. These results illustrate that the AMH concentration is a useful marker for predicting embryo production after superovulation, and donors with high AMH levels produce more transferable embryos at low FSH doses than at high doses.

Key words: Anti-Müllerian hormone, Embryo production, Follicle-stimulating hormone dose, Japanese Black cattle, Ovarian response

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The use of technologies focused on the intensive production of various livestock breeds, including Japanese Black (JB) cattle, is considered important in traditional breeding and embryo transfer programs to enhance their breeding ability [1, 2]. The ability to produce embryos is a reproducible and heritable trait utilized in multiple ovulation embryo transfer (MOET) and oocyte pickup and *in vitro* production (OPU−IVP) programs [2−4]. Anti-Müllerian hormone (AMH) is a valuable endocrine marker for predicting embryo production potential in cattle in both MOET and OPU−IVP programs [3, 4]. We previously reported the efficacy of a single measurement of serum AMH levels in 7−10-month-old JB heifers to predict their responses to gonadotropin treatment at the heifer stage, select superior embryo donors within a herd by excluding animals with a low response, and establish protocols for improving reproductive performance within the herd [1, 2]. Although MOET technology has improved productivity and reproductive performance at cattle production sites worldwide, large variations observed in excess ovulation performance among individual cows following follicle-stimulating hormone (FSH) treatment, possibly due to environmental, feeding, and stress-related factors [4−6], remain major barriers to the success of MOET and OPU−IVP programs. Moreover, there is an inverse correlation between the number of ovarian follicles and blood FSH concentrations, and excessive FSH administration during superovulation is detrimental to ovulatory follicle function, ovulation rate, oocyte quality, and embryo survival in cattle [7−10]. Between June 2022 and October 2023, the seasons of superovulation treatment were classified into four categories: spring (n = 34, March–May), summer (n = 33, June–August), autumn (n = 27, September–November), and winter (n = 30, December–February). The number of embryos recovered from donors in spring, summer, autumn, and winter were 14.8 ± 1.7, 15.5 ± 1.4, 16.7 ± 1.7, and 16.9 ± 2.6, respectively. The number of transferable embryos recovered from donors in spring, summer, autumn, and winter were 9.2 ± 1.4, 9.8 ± 1.2, 8.9 ± 1.6, and 10.2 ± 1.5, respectively. There were no significant differences between seasons. Since AMH is detected in the granulosa cells of all developing follicles, with the highest expression in healthy follicles of 3−7 mm [11], we hypothesized that the optimal FSH dose in a superovulation program may depend on the donor's AMH levels, follicle count, or both. Therefore, the FSH dose should be adjusted, either increased or decreased, on an individual basis according to their AMH concentrations. Serum AMH concentration is a moderately heritable trait and is negatively affected by factors such as inadequate maternal nutrition during early pregnancy [12]. We previously reported considerable variations in the AMH concentrations of sister heifers derived from the same parents in a MOET program using 10 JB donor cows [1]. As a valuable genetic resource, JB cattle are frequently subjected to superovulation treatments to enhance their breeding ability, despite having low AMH concentrations. Consequently, there is a strong demand for an FSH-induced superovulation treatment protocol that can maximize the response of ovarian antral follicle (AF) in JB donors to obtain a large number of transferable embryos. Therefore, it was necessary to determine the optimal FSH dose for each donor. Prior research has not documented any findings regarding a correlation

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between AMH concentrations in donors and different FSH doses used in a superovulation program, specifically concerning the ovarian response in JB cattle. We hypothesized that the AMH concentrations of donors would affect their ovarian response to different doses of FSH in superovulation treatments. This study aimed to assess the correlation between donor AMH levels and the different FSH doses used in a superovulation program. Herein, the ovarian responses of donors who participated in a superovulation program conducted in JB cattle were retrospectively analyzed. In addition, data regarding their AMH concentrations and corresponding FSH doses were considered to investigate the relationships among these factors.

Serum AMH concentrations of 34 JB cows used in this superovulation program were in the range of 343−2,806 pg/ml, and their mean and median AMH concentrations were 1,483.3 ± 113.0 pg/ml and 1,465 pg/ml, respectively. The cows were classified into four groups based on their median AMH concentrations (AH: ≥ 1,465 pg/ml, AL: < 1,465 pg/ml), and the FSH doses (FH: 16−18 AU, FL: 14−15 AU) used in the program were as follows: AH-FH (AMH: ≥ 1,465 pg/ml, FSH: 16−18 AU, n = 39), AH-FL (AMH: ≥ 1,465 pg/ml, FSH: 14−15 AU, n = 27), AL-FH (AMH: < 1,465 pg/ml, FSH: 16−18 AU, n = 39) and AL-FL (AMH: < 1,465 pg/ml, FSH: 14−15 AU, n = 19), where *n* represents the total number of superovulation treatments performed on the donors in each group. A histogram of AMH concentrations and group composition based on the median AMH concentrations and two FSH doses is shown in Fig. 1. The ages of the examined JB cows at the time of blood collection in the AH-FH, AH-FL, AL-FH, and AL-FL groups were 9.7 ± 0.5, 11.2 ± 0.6, 10.2 ± 0.6, and 11.2 ± 0.8 years, respectively, with no significant differences among the groups. The ages of donors at the time of superovulation treatments in the AH-FH, AH-FL, AL-FH, and AL-FL groups were 9.8 ± 0.5, 11.3 ± 0.6, 10.2 ± 0.6, and 11.3 ± 0.8 years, respectively, with no significant differences among the groups. The number of superovulation treatments of donors in the AH-FH, AH-FL, AL-FH, and AL-FL groups was 2.8 ± 0.2, 2.8 ± 0.3 2.9 ± 0.3, and 3.1 ± 0.3, respectively, with no significant differences among the groups.

The differences in the number of total and transferable embryos recovered from the four groups were analyzed. The number of total and transferable embryos recovered in the present study are shown in Fig. 2. The total number of embryos recovered from the donors in the AH-FH, AH-FL, AL-FH, and AL-FL groups were 17.8 ± 1.8, 20.6 ± 2.0, 14.3 ± 1.4, and 8.6 ± 1.7, respectively. Notably, the number of embryos recovered from the AH-FH and AH-FL groups was significantly higher than that recovered from the AL-FL group ($P < 0.001$ and $P = 0.006$, respectively), and the number of embryos recovered from the AL-FH group was higher than that recovered from the AL-FL group ($P = 0.052$). The number of transferable embryos recovered from the donors in the AH-FH, AH-FL, AL-FH, and AL-FL groups were 9.2 ± 1.2, 13.0 ± 1.8, 9.4 ± 1.1, and 5.6 ± 1.4, respectively, with the number of transferable embryos recovered from the AH-FL groups being significantly higher than that recovered from the AL-FL group ($P = 0.007$).

Thus, the total number of embryos recovered from donors in groups with high AMH levels was higher than that obtained from donors with low AMH levels. In particular, the total number of embryos recovered from the AH-FH and AH-FL groups was significantly higher than that recovered from the AL-FL group, which is in agreement with previous studies [2, 5]. Notably, although there were no significant differences, the total number of recovered embryos did not decrease at lower FSH doses in the groups with high AMH levels (AH-FH and AH-FL), whereas the groups with low AMH levels (AL-FH and AL-FL) exhibited a decrease in the total number of recovered embryos with a reduction in FSH dose. Mossa *et al*. [13] reported that although the mechanism remains unclear, circulating FSH levels were lower in cows with high AF counts (AFC) than in cows with low AFC, which may indicate that cows with high AMH levels are relatively more sensitive to FSH levels in terms of follicle development than those with low AMH levels. In the present study, the administration

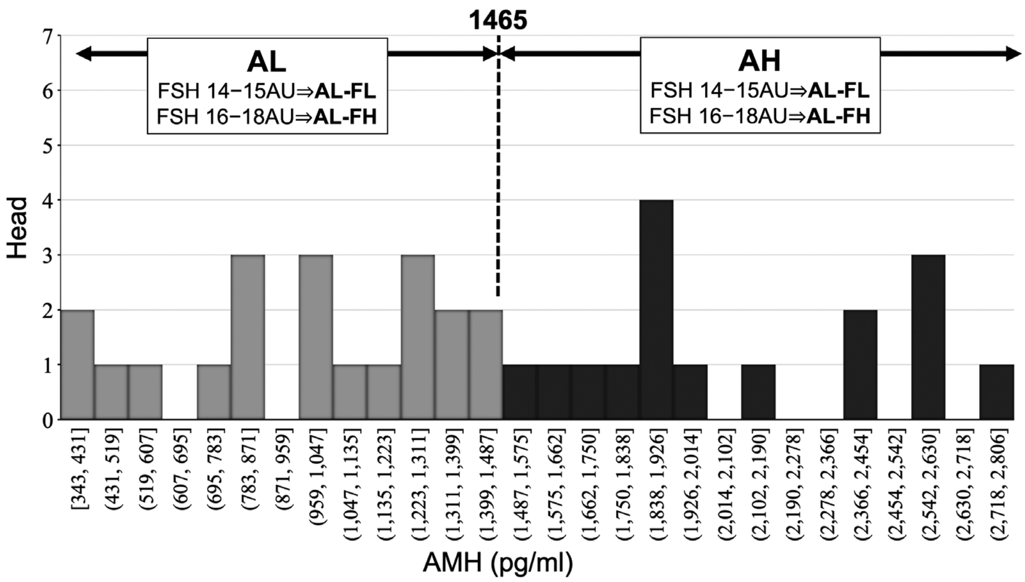

Fig. 1. Frequency distribution of serum concentrations of anti-Müllerian hormone (AMH) in the donor cows (N = 34), and study group classification based on serum AMH concentration and administered doses of follicle-stimulating hormone (FSH). AH-FH (AMH: ≥ 1,465 pg/ml, FSH: 16−18 AU, n = 39), AH-FL (AMH: ≥ 1,465 pg/ml, FSH: 14−15 AU, n = 27), AL-FH (AMH: < 1,465 pg/ml, FSH: 16−18 AU, n = 39), and AL-FL (AMH: < 1,465 pg/ml, FSH: 14−15 AU, n = 19). Note: N represents the number of donor cows used in the superovulation program; n represents the total number of superovulation treatments performed on donors in each group.

of low doses of FSH to cows with high AMH levels did not reduce the total number of recovered embryos, supporting the idea that AF in cows with high AMH levels is relatively more sensitive to FSH levels. However, cows with low AMH levels may respond to increased exogenous FSH doses by increasing the number of AFs responding to this stimulus. However, there was a significant difference in the number of transferable embryos recovered from the AH-FL and AL-FL groups in the present study. These results suggest that there may be an appropriate FSH dose that reflects the changes in AMH concentrations in donors and that excessive FSH concentrations are detrimental to the normal development of AF and oocyte maturation. Generally, the ovarian response depends on AMH levels in donors at constant endogenous FSH levels; however, the results of the present study confirm that exogenous FSH doses also affect the ovarian response in donors in response to changes in AMH levels.

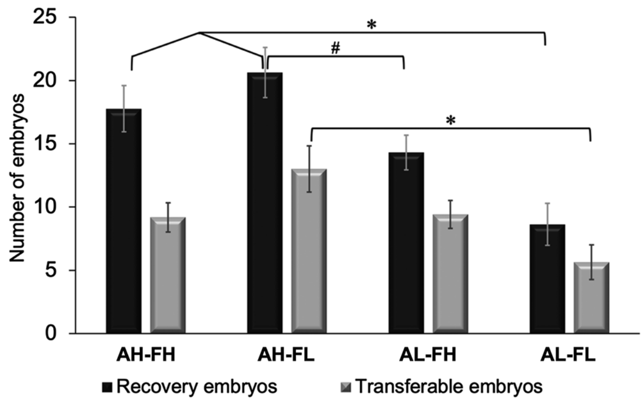

Fig. 2. Number of recovered and transferable embryos from the donor cows. * indicates significant differences ($P < 0.05$), # tends to exhibit a significant difference ($0.05 < P < 0.1$). Abbreviations: AMH, anti-Müllerian hormone; AH-FH (AMH: ≥ 1,465 pg/ml, FSH: 16−18 AU, n = 39), AH-FL (AMH: ≥ 1,465 pg/ml, FSH: 14−15 AU, n = 27), AL-FH (AMH: < 1,465 pg/ml, FSH: 16−18 AU, n = 39) and AL-FL (AMH: < 1,465 pg/ml, FSH: 14−15 AU, n = 19).

Subsequently, the relationship between AMH concentrations and the total number of recovered and transferable embryos obtained from each of the four groups were analyzed (Fig. 3). A significant positive correlation was observed between the AMH concentration and the number of embryos recovered from the AH-FH (n = 39, P = 0.014, r = 0.39) and AL-FH (n = 39, P < 0.01, r = 0.45) groups. A significant positive correlation was also observed between the AMH concentration and the number of transferable embryos recovered from the AL-FH group (n = 39, P = 0.03, r = 0.35). These results suggest that cows with low AMH concentrations in the present study had increased total recovered and transferable embryos at high FSH dosages compared to those at low FSH dosages, implying that the observed positive correlation with AMH levels may be used to predict embryo production in field practice. However, it was challenging to assess the appropriateness of the two FSH doses used in this study in herds with high AMH concentrations; therefore, both higher and lower FSH doses than those used in this study must be tested.

Superovulation is an important technique for obtaining multiple

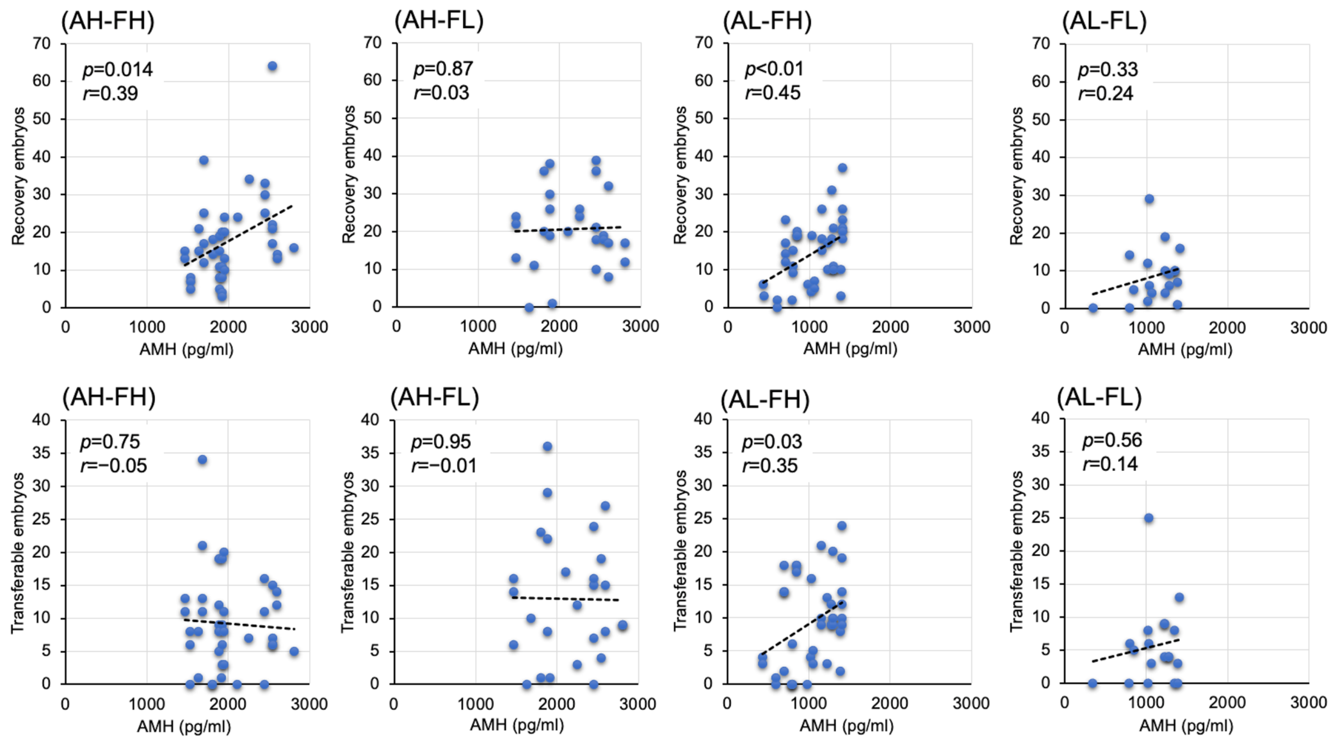

Fig. 3. Correlation between the number of embryos and serum AMH concentrations in donors. The upper figures show the correlation between the total number of recovered embryos and serum AMH concentrations (AH-FH, n = 39; AH-FL, n = 27; AL-FH, n = 39; and AL-FL, n = 19). The lower figures show the correlation between the number of recovered transferable embryos and serum AMH concentrations (AH-FH, n = 39; AH-FL, n = 27; AL-FH, n = 39; and AL-FL, n = 19). Note: n represents the total number of superovulation treatments performed on donors in each group. AMH, anti-Müllerian hormone; AH-FH (AMH: ≥ 1,465 pg/ml, FSH: 16−18 AU); AH-FL (AMH: ≥ 1,465 pg/ml, FSH: 14−15 AU); AL-FH (AMH: < 1,465 pg/ml, FSH: 16−18 AU); and AL-FL (AMH: < 1,465 pg/ml, FSH: 14−15 AU).

embryos from excellent dams, and is practiced worldwide in the field of breeding management in the cattle industry. Several factors affect embryo production, such as the hormone type and dosage used for superovulation, the age and AFC of donor animals, as well as feeding management, herd nutrition, and stress-related factors, and research has been conducted to address these issues to obtain good embryo recovery results [4, 15]. Serum AMH concentrations have served as valuable endocrine markers for predicting embryo production in cattle in both MOET and OPU−IVP programs, including JB cattle [3−5, 14]. We previously reported the usefulness of a single AMH concentration measurement for selecting JB heifer embryo donors for herd breeding programs [1, 2]. Although AMH concentrations are good predictive markers for the number of embryos that can be recovered, they are not associated with the number of transferable embryos. Studies have indicated that AMH concentrations are highly variable among individuals and that factors affecting ovarian reserves (indirectly affecting AMH concentrations) may include maternal nutrition, disease conditions, and the presence of endocrine disrupters during pregnancy [12, 16, 17]. In our previous study, although some sister heifers from the same donor parents had similar serum AMH concentrations, others exhibited low AMH concentrations for same nutritional conditions [1]. In the JB herd, when donors are selected based on their AMH concentrations, which indicates their embryo production potential [1, 2], even donors with low AMH concentrations may be selected based on genetic values for meat quality, growth rate, and frame type; thus, cows with various AMH concentrations are often subjected to superovulation treatments. Therefore, there is a strong need for FSH-induced superovulation treatment methods to maximize the response of the ovarian AF of JB donors and to obtain large number of transferable embryos for donors with various AMH concentrations. Our results demonstrated the potential of a new FSH administration method that can maximize donor performance in MOET and OPU−IVP programs in herds with various AMH concentrations. In the present study, the median AMH concentration was used as the cut-off value to classify the groups. In the future, relatively larger number of cows should be used, AMH concentrations should be based on quartiles, and the results of superovulation treatments should be compared using higher and lower FSH doses than those used in this study.

It has been shown that despite a significant positive correlation between plasma AMH concentrations and the number of recovered embryos, repeated superovulation in JB cows results in a decrease in the average AMH concentrations and the number of embryos, suggesting that the prediction accuracy of ovarian response based on the first measurement of AMH concentrations decreases during repeated superovulation [5]. In this study, the AMH concentrations of donors were measured once after their introduction to the farm, and a retrospective data analysis was conducted using the recorded data from 124 superovulation trials based on these AMH concentrations. Therefore, a decrease in the AMH concentration was assumed during these repeated superovulation trials, which can be considered a limitation of the data presented in this study.

In conclusion, these results confirm that AMH concentration is a useful predictive marker for the number of recovered embryos, as reported previously. Furthermore, donors with high AMH concentrations produced more transferable embryos at low FSH doses than at high FSH doses. However, high FSH doses may increase the number of transferable embryos recovered from cows with low AMH concentration.

Methods

All experiments were conducted in accordance with the regulations for the protection of experimental animals and guidelines of Yamaguchi University, Japan (no. 40, 1995; approval date: March 27, 2017). Informed consent was obtained from all farmers.

The investigation was conducted with 34 JB donors (mean ± standard error of the mean, 10.4 ± 0.3 years; range: 3.0−15.5 years at the time of blood collection) at a commercial farm in Kagoshima, Japan, between June 2022 and October 2023. Venous blood samples were collected from the donors once after their introduction to a commercial farm, and their serum AMH levels were measured to predict the ovarian response to superovulation treatments conducted between September 2022 and April 2023. The blood samples were collected in silicone-coated tubes, and were centrifuged at 3000 × *g* for 10 min to recover the serum, which was stored at −20°C until the AMH assays were performed. Serum AMH concentrations were measured using a commercially available ELISA kit (AnshLabs, Webster, TX, USA) [1, 2].

All 34 JB donors underwent 1–8 superovulation treatments, with 124 superovulation treatments performed between June 2022 and October 2023. Superovulation was induced in animals using FSH (Antrin®R-10; Kyouritsu Seiyaku, Tokyo, Japan), which was administered twice daily in decreasing doses of 14−18 AU over 3 days. The total dose of FSH used in this study was based on a range of FSH doses that have been empirically found to have no significant effect on the number of recovered and transferable embryos, based on embryo recovery records obtained from previous superovulation treatments in the study herds.

An injection of prostaglandin $F_{2\alpha}$ (cloprostenol, 500 µg/cow; Resipron®-C; ASKA Animal Health, Tokyo, Japan) was administered twice on day 3 of superovulation treatment. A controlled internal drug release device (CIDR 1900; Zoetis Japan, Tokyo, Japan) was inserted into the cows for 7 days until prostaglandin $F_{2\alpha}$ injection. An injection of estradiol benzoate (1 mg/cow; Ovahormon®; ASKA Animal Health, Tokyo, Japan) was administered 4 days before the first FSH injection. Donors were administered a single dose of 100 µg of GnRH (Fertirelin acetate, 100 µg/cow; CONSULTAN ®INJECTION; ASKA Animal Health, Tokyo, Japan) 24−32 h after second administration of prostaglandin $F_{2\alpha}$, inseminated 12−24 h after GnRH administration, and embryos were recovered 7 days after the insemination. The recovered embryos were classified according to the criteria of the International Embryo Technology Society. "Transferable embryos" included embryos with codes 1–3, and "recovery embryos" included transferable embryos plus unfertilized oocytes with code 4 (dead or degenerating). The protocol for the superovulation program is presented in Supplementary Fig. 1.

The results are expressed as mean ± standard error of the mean. Statistical analyses were performed using the Bell Curve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan). The data obtained from the parameter analyses were analyzed using one-way analysis of variance and Tukey−Kramer's *post hoc* test. The association between AMH concentration and the total number of recovered and transferable embryos was evaluated using Pearson's correlation coefficient. A P-value < 0.05 was considered statistically significant, whereas *p*-values in the range of 0.05−0.1 were considered to indicate a trend towards significance.

Conflict of interests: The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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