Prediction of Superovulatory Response in Japanese Black Cattle Using Ultrasound, Plasma Anti-Müllerian Hormone Concentrations and Polymorphism in the Ionotropic Glutamate Receptor AMPA1/GRIA1

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Abstract. The aim of this study was to improve the reliability of predicting the superovulatory response in Japanese Black cattle. Follicle counts and plasma anti-Müllerian hormone concentrations were analyzed within four days prior to the initiation of superovulation. The single nucleotide polymorphism (guanine or adenine) of the ionotropic glutamate receptor AMPA1 was determined. The plasma anti-Müllerian hormone concentration was positively correlated (P<0.001) with the numbers of all follicles and small (<5 mm) follicles and with the numbers of ova/embryos (P<0.001), fertilized embryos (P<0.001) and transferable embryos (P=0.005). There was no significant difference in follicle counts and superovulatory responses between donor cows bearing guanine/adenine or guanine/guanine alleles of AMPA1. Donor cows with a high plasma anti-Müllerian hormone concentration and homozygous for the guanine-containing allele of AMPA1 were most responsive to superovulation. The results suggest that physiological and genetic markers of superovulation have a synergistic effect on the accuracy of predictions of responsiveness.

Key words: AMH, AMPA1, GRIA1, Japanese black cattle, Superovulation

In cattle, which are single-ovulating species, superovulation is an important technique to obtain multiple embryos from an excellent dam. However, considerable individual variability in the responsiveness to superovulation treatment has been a limiting factor affecting embryo production efficiency [1]. Improvements in the reliability of superovulation are key to genetic advancement programs using embryo transfer technology. Although in the past only results of superovulation treatment in the same individual were applicable, physiological and genetic markers of the efficiency of embryo production have been reported recently. This study was conducted to investigate whether a combination of these markers could improve the reliability of predictions of the superovulatory response in Japanese Black cattle.

Numbers of ovarian follicles are positively associated with superovulatory response in cattle [2–4]. Although ultrasound testing of ovaries is one method of preselecting donor cows, operating an ultrasound device requires a certain amount of skill. Anti-Müllerian hormone (AMH) is a glycoprotein belonging to the transforming growth factor beta family and is produced by granulosa cells of healthy growing follicles [5]. In assisted reproductive technologies, a low plasma AMH concentration is used as a criterion for ovarian aging [6]. The circulating AMH concentration is an endocrine marker that allows the prediction of reserves of antral follicles in the ovary and response to ovarian stimulatory treatment in humans [7, 8] and cattle [9, 10].

Genetic approaches have also been used to regulate the ovarian response to superovulation in cattle. Recently, it was reported that a single nucleotide polymorphism (SNP) of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1 (AMPA1/GRIA1) in cattle correlated with an increased number of ovulations [11]. AMPA1 is an ionotropic transmembrane receptor for glutamate and mediates excitatory neurotransmission by opening ion channels upon the binding of glutamate [12]. The SNP, adenine for guanine, in exon 7 replaces a serine with asparagine at amino acid residue 306 in AMPA1. It has been suggested that the polymorphism in AMPA1 affects the number of ovulations by controlling the secretion of luteinizing hormone because the single amino acid substitution changes the affinity of the receptor for glutamate.

As shown in Table 1, plasma AMH concentrations were positively correlated (P<0.001) with the total number of follicles and the numbers of small follicles, AMH concentrations were positively correlated with the numbers of ova/embryos (P<0.001), fertilized embryos (P<0.001) and transferable embryos (P=0.005). The number of small follicles showed a significant correlation with the numbers of ova/embryos (P<0.001), fertilized embryos (P<0.001) and transferable embryos (P<0.001). When donor cows were divided into low (L) and high (H) groups on the basis of the average plasma AMH concentration (0.21 ng/ml), the H group showed a significantly higher number of ova/embryos after superovulation than the L group (Fig. 1). These results suggested that both the plasma AMH concentration and the number of ovarian follicles are useful in the prediction of embryo production capacity. On the other hand, the plasma AMH concentration and the number of small follicles did not correlate with the proportion of fertilized embryos and transferable embryos.
Therefore, the numbers of fertilized embryos and transferable embryos increased in parallel with increased number of ova/embryos, and the quality of embryos did not vary in accordance with differences in superovulatory responses.

In the present study, the 12 donor cows that received multicycle superovulation treatments could be divided into two groups. The plasma AMH concentrations of donors A to G and donors H to L were always lower or higher than the limit of detection of the assay, respectively (Fig. 2). Therefore, the plasma AMH concentration seemed to be an indicator of response for long periods if measured once before superovulation. Follicle counts and plasma AMH concentrations were well correlated. However, there were individual cases (e.g., donor J) where follicle counts were better than plasma AMH concentrations as an indicator of embryo production. Therefore, analysis of follicle counts could strengthen predictions based on AMH levels.

In this study, we performed superovulation treatments with no synchronization of the follicular wave. Furthermore, follicular counts were made for 4 days before the first injection of follicular stimulating hormone (FSH). It is well established that a greater superovulatory response is obtained when treatment is initiated once the follicular wave has emerged [13]. The number of follicles detected on given days of the estrus cycle was not always enough to estimate the number of follicles on the day the wave emerged [4]. Therefore, synchronization of the follicular wave might further enhance the reliability of prediction of superovulatory responses based on follicular counts.

We measured plasma AMH concentrations without regard for the estrus cycle because the majority of the donor cows were treated with a program using CIDR. Nevertheless, the AMH level was a usable marker of superovulatory responses. In human, nonsignificant variations of circulating AMH concentrations throughout the menstrual cycle have been reported, and the circulating AMH concentration is utilized as a marker of ovarian reserve, which can be measured anytime during the menstrual cycle [14]. Although there are reports that have found significant cyclical fluctuations in circulating AMH concentrations with a decrease after ovulation, these variations are similar to inter-cycle variability [15, 16]. In cattle, circulating AMH concentrations were constant during the 6- to 9-day breeding period prior to ovulation [9]. Another study showed that circulating AMH concentrations followed a specific dynamic profile during the estrus cycle with the highest correlations between the number of ovulations and AMH levels at estrus and after Day 12 of the cycle [17]. In any case, these studies suggest that circulating AMH concentrations are useful for selecting donor cows able to produce large numbers of embryos for an extended period of the cycle.

Out of 34 donor cows, 13 and 21 had the guanine/adenine (GA) and guanine/guanine (GG) alleles of AMPA1, respectively. The adenine/adenine type was not included in this experiment. The gene frequency of the G allele was 0.809. There was no significant difference in follicle counts and superovulatory responses between donor cows bearing GA and GG alleles (Fig. 3). As shown in Fig. 4, donor cows were categorized into four groups on the basis of plasma AMH concentrations (L or H) and AMPA1 type (GA or GG). H-GG (n=9) showed significantly higher numbers of ova/embryos and fertilized embryos than L-GA (n=13) and L-GG (n=20). The number of transferable embryos was significantly higher in H-GG...
than in L-GA, L-GG and H-GA (n=7).

In the present study, donor cows bearing GG alleles of AMPA1 and higher plasma AMH levels were most responsive. Despite the fact that no donor cows homozygous for the A allele were included in this study, the selection of cattle homozygous for the G allele appears to improve the inherent capacity for embryo production in herds. The precise mechanism by which AMPA1 affects superovulation is still unclear. A previous study suggested that the polymorphism of AMPA1 affects the secretion of luteinizing hormone through the regulation of GnRH release [11]. However, cows homozygous for the G allele in that study had significantly higher numbers of small follicles than cows homozygous for the A allele on the day preceding the initiation of superovulation treatment. Although we could not compare follicle counts in cows homozygous for the G and A alleles, the SNP in AMPA1 may be involved in the development of ovarian follicles.

In summary, the present study revealed that a combination of physiological and genetic markers has a synergistic effect on the reliability of predictions of superovulatory response. These markers could be exploited in the preselection of donor cows used for superovulation and the genetic improvement of herds.

Methods

Animals and treatments

Thirty-four Japanese Black cattle, 3–17 years old, were kept in the animal research center and induced to superovulate with FSH (20 IU/cow, Antorin®R-10, Kawasaki Seiyaku, Kanagawa, Japan) given twice daily in decreasing doses over 3 days. Twelve donor cows were used for superovulation two to four times, and a total of 49 superovulation treatments were carried out. An injection of prostaglandin F$_{2\alpha}$ (cloprostenol 0.5 mg/cow, Resipron®-C, ASKA Pharmaceutical, Tokyo, Japan) was given on the 3rd day of superovulation treatment. All but three donor cows had a controlled internal drug release device (CIDR® 1900, Pfizer Japan, Tokyo, Japan) inserted for 8 to 10 days until the injection of prostaglandin F$_{2\alpha}$. The CIDR was randomly inserted some time 2 days after estrus. Three donor cows were treated with FSH from 6 to 8 days after estrus without a CIDR. Donor cows were inseminated 24 h after the onset of estrus,
and embryos were recovered 7 to 8 days after insemination.

The collection of venous blood samples and counting of follicles were performed within four days prior to the initiation of superovulation. Plasma samples of all donor cows were harvested by centrifugation and stored at –20 C prior to use. Ovaries from a total of 46 donor cows were analyzed by an ultrasound imaging device using a transrectal probe (SSD-900, 7.5 MHz, Aloka, Tokyo, Japan) to measure follicular size. The follicles were counted and allocated to three classes, small (<5 mm), medium (5–10 mm) and large (>10 mm).

**Plasma AMH concentration**

AMH concentrations were measured with an AMH Gen II ELISA (Beckman Coulter, Brea, CA, USA). Undiluted plasma (20 μl) was used for the analysis. Plasma AMH concentrations were lower than the limit of detection of the assay (0.08 ng/ml) in 26 of 49 samples tested. Values for these samples were taken as 0 ng/ml. AMH was undetectable in plasma from castrated animals.

**Polymorphism in AMPA1**

The polymorphism of AMPA1 was determined according to a previous report [11]. Briefly, 241 bp of exon 7 were amplified by PCR using the primers GRIA1 exon7F (AGCCTCCTACACAGCTCTCT) and GRIA1 exon7R (CGTTGTGCAGGCTCAC). Direct DNA sequencing of the PCR products was performed using an ABI PRISM3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The SNP (guanine/adenine) at nucleotide position 111 was identified.

**Statistical analysis**

All results are presented as the means ± SEM. The relationships between plasma AMH concentrations, results of embryo production and follicular counts were evaluated using Pearson’s correlation coefficient. The experimental data were analyzed using the glm() function of the R statistical package [18]. Multiple comparison tests were conducted using the glht() function. A P value <0.05 was considered significant.

**References**