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Original

Serum concentration of anti-Müllerian hormone and its relationship with ovarian reserve in Brahman oocyte donors

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ABSTRACT

Objetive. To evaluate the relationship of AMH blood concentration with ovarian follicular count and *in vitro* embryo production in female Brahman cattle. **Material and methods.** To standardize the AMH quantification for Brahman oocyte donors, experiment 1 was performed, blood samples were taken from 10 heat synchronized Brahman females, in three different days of the estrous cycle, with more than 90 days postpartum and with normal reproductive evaluation. Serum concentration of AMH was determined with a commercial immunoenzymatic kit. In experiment 2, blood samples were taken from 100 non-synchronized Brahman oocyte donors, an ovum pick-up session was performed for *in vitro* embryo production and the number of follicles greater than 2 mm in the two ovaries was registered. **Results.** There were no differences in AMH concentration between the evaluated days of estrous cycle and a correlation of 0.82 ($p<0.001$) was found between antral follicle population (AFP) and AMH concentration. Serum AMH concentration ranged from 0.02 to 2.69 ng/ml in Brahman oocyte donors. Also, a correlation of 0.73 ($p<0.001$) between AMH and AFP and 0.54 between the AMH and the percentage of blastocysts were found in donors. **Conclusions**. The AMH can be used as a satisfactory endocrine marker of ovarian reserve prediction for *in vitro* embryo production in Brahman cattle.

Keywords: Zebu; follicle; *in vitro*; endocrine marker; embryo transfer (*Source: MeSH*).

RESUMEN

Objetivo. Evaluar la relación de la concentración sanguínea de AMH con el recuento folicular ovárico y la producción *in vitro* de embriones en hembras bovinas de la raza Brahman. **Materiales y métodos.** Para estandarizar la técnica de cuantificación de AMH se realizó un primer experimento, en el cual se tomaron muestras de sangre de 10 hembras Brahman sincronizadas en su celo, en tres días diferentes del ciclo estral, con más de 90 días posparto y con evaluación reproductiva normal. La concentración sérica de AMH se determinó con un *kit* inmunoenzimático comercial. Una vez estandarizada la técnica, se realizó un segundo experimento, se tomaron muestras de sangre de 100 donantes Brahman de ovocitos no sincronizadas, se realizó una sesión de aspiración folicular para la producción *in vitro* de

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embriones y se registró el número de folículos mayores de 2 mm en los dos ovarios. **Resultados.** No hubo diferencias en la concentración de AMH entre los días evaluados del ciclo estral y se encontró una correlación de 0,82 ($p<0.001$) entre la población de folículos antrales (PFA) y la concentración de AMH. La concentración sérica de AMH osciló entre 0.02 y 2.69 ng/ml. Además, se encontró una correlación de 0.73 ($p<0.001$) entre AMH y AFP y 0.54 entre AMH y el porcentaje de blastocistos producidos. **Conclusiones.** La AMH se puede utilizar como un marcador endocrino satisfactorio de la predicción de la reserva ovárica para la producción de embriones *in vitro* en ganado Brahman.

Palabras clave: Cebú; folículo; *in vitro*; marcador endocrino; transferencia embrionaria (*Fuente: MeSH*).

INTRODUCTION

In order to increase the efficiency of livestock as a production system, bovine assisted reproduction technologies such as follicular aspiration or OPU (ovum pick-up) and *in vitro* embryo production (IVEP) are important biotechnologies to multiply the genetic material of animals with reproductive and productive superiority (1). However, the success in embryo production, among other factors, depends on individual physiological characteristics of the oocyte donor, such as the ovarian antral follicle population (AFP), as well as the quality and quantity of recovered oocytes (2,3,4). Female mammals are born with a variable number of healthy follicles in their ovaries (5), they constitute the ovarian reserve from which primordial follicles will be activated and recruited into the pool of growing follicles to eventually undergo atresia or ovulation (6).

The anti-Müllerian hormone (AMH), is a glycoprotein hormone, also called Müllerian-inhibiting substance (MIS), is a member of the superfamily of transforming growth factors beta (TGF-β) (7).

In males, this hormone is secreted during the fetal phase in the sexual differentiation (7) and in the female it is secreted by granulosa cells of small antral follicles, which has been reported in various species, such as human, bovine and ovine (8,9,10). In adult life, AMH plays a role in folliculogenesis, during the recruitment and selection process, regulating follicles growth, by participating in the control of follicle-stimulating hormone (FSH) release (7,11). The secretion pattern was first evaluated in rodents (12) and later in women (13), cattle (14) and sheep (15).

The AMH is considered an excellent endocrine marker of ovarian reserve and ovarian response to gonadotropin stimulation in humans (16). Subsequently, this correlation has been extended to some domestic species (17,18), in such a way that this marker can predict AFP in cattle (19,20,21). Studies with different cattle breeds, such as Holstein, Jersey, Gyr and Nelore, show the relationship between AMH and number of follicles (17,19,22,23,24).

The hormone has also been proposed as a marker to predict the performance of IVEP in *Bos taurus* (25,26) and *Bos indicus*, cattle breeds (27,28,29). In this way, AMH as an endocrine marker of AFP can be useful to select donor females with high reproductive potential and increase embryo production efficiency (22,30).

Several studies (18,20,21,24) have stated that one of the practical advantages of using AMH over direct counting of follicles with ultrasound to predict AFP, is that AMH levels may not present greater variations during the estrous cycle, therefore, blood samples can be taken at any time of the cycle to evaluate circulating concentrations of AMH, however, this has not been reported in Brahman donor cows.

Despite being *Bos indicus,* the Brahman breed was developed by north American breeders, crossing Guzerat, Nelore, Gyr and Krishna Valley, at the end of the 19th and beginning of the 20th century aiming to obtain a breed adapted to tropical environmental conditions (31). In Colombia, it is the breed with the highest participation as a pure beef breed (97%) and with great influence on cattle commercialization (32).

Therefore, the objective of this study was to evaluate the relationship of AMH blood concentrations with the ovarian reserve and IVEP in oocyte donor cows of the Brahman breed in Colombia.

MATERIAL AND METHODS

Study site. The present study was developed in farms in the municipalities of Villavicencio, Granada, Cabuyaro, Yopal, Girardot, Oiba, Alvarado, Purificación and Espinal in Colombia.

The laboratory analysis was carried out at the facilities of the Embriogenex Animal Genetics and Reproduction Biotechnology Center and at the laboratories of the La Salle University located in Bogotá. The study was approved by the La Salle University Research Ethics Committee (Approval #235 of 2017).

Standardization of AMH blood detection technique in Brahman cows (experiment 1). Ten Brahman cows, oocyte donors of a commercial herd located at the municipality of Purificación, in Tolima state, Colombia, were used. The females were between 4 and 5 years old, multiparous, and at the time of the experimental procedures had more than 90 days postpartum, normal reproductive evaluation, were not pregnant, and did not present pathologies in their two ovaries.

In order to validate if there were differences in blood concentration of AMH according to the day of the estrous cycle, females were heat synchronized: on day zero (d0) an intravaginal device of 1.0 g of natural progesterone was inserted (DIB 1.0, Syntex SA, Buenos Aires, Argentina) and 2 mg of estradiol benzoate, im (Estradiol Benzoate, Syntex SA, Buenos Aires, Argentina) was administered. At the time of device removal (d8) 0.15 mg of D-cloprostenol (Prostal®, Laboratorios Over, Santa Fé, Argentina) and 1 mg of estradiol cypionate (Cipiosyn, Syntex S.A., Buenos Aires, Argentina) were applied im. Blood samples to determine serum AMH concentrations were taken on days 8, 13 and 23 in relation to the day of insertion of the device (d0), aiming to evaluate follicular and luteal phase, and transrectal ultrasound was performed using a 5 MHz convex transducer (Mindray DP – 2200 VET, Shenzhen, China) and the number of visible antral follicles > 2 mm in diameter was determined in both ovaries (33).

Blood samples were collected in a vacuum tube containing EDTA by puncturing the coccygeal vein. Samples were kept refrigerated until separation from the blood plasma by centrifugation at 3600 g for 10 minutes and stored at -70°C until analysis.

To evaluate blood AMH concentrations, an enzyme-linked immunosorbent assay (ELISA) was performed with a commercial kit available for bovine AMH (AL-114; Ansh Labs, Webster, Tx, USA- with a sensitivity of 0.1 ng/ml and a detection limit of <0.078 ng/ml) following the manufacturer's instructions. A microplate reader (Mindray MR-96A) was used. For standardization, each sample was measured in triplicate and in three different assays to obtain intra- and inter-assay variation.

Determination of AMH in Brahman oocyte donors (experiment 2). For this experiment, Brahman cows, oocytes donors (n=100), were selected from a pool of herds that perform commercial IVEP with the Embriogenex© company. All females were between 4 and 7 years old, multiparous, at the time of the experimental procedures had more than 90 days postpartum, normal reproductive evaluation, were not pregnant, did not present pathologies in their two ovaries and were not submitted to estrus synchronization. Blood samples were taken before starting the commercial OPU session, following the same procedures previously described.

Before the OPU sessions, all ≥3 mm antral follicles in both ovaries were counted (34)AFC using an ultrasound machine with a 5 MHz transrectal probe. For OPU procedures, the donors had their movements restricted in a chute and received an epidural anesthesia (2% lidocaine) to facilitate ovaries handling. The perineal area was cleaned, dried and disinfected with alcohol. All visible follicles were aspirated through an aspiration needle (20 G; Terumo Europe NV, Belgium) installed inside a transvaginal probe and connected to a vacuum system (85-90 mm Hg negative vacuum pressure; V-MAR 5000, Cook Australia, Queensland, Australia). The follicular fluid was conducted through a hose circuit with an internal diameter of 1.1 mm and 120 cm long (Watanabe Tecnologia Aplicada, WTA Ltda, Cravinhos, São Paulo, Brazil) connected directly to a 50 ml conical tube containing 15 ml of Dulbecco's buffered phosphate saline solution (DPBS; Nutricell Nutrientes Celulares, Campinas, São Paulo, Brazil) and 5000 IU/ml sodium heparin, kept at 37°C. Oocytes were recovered and sorted in DPBS medium supplemented with 1% fetal bovine serum and then transferred to 1.5 ml tubes containing oocyte transport medium [TCM 199 with 25 mM HEPES and Earle's salts (M7528)] supplemented with 10% FCS fetal serum, 49.4 mg/ml sodium pyruvate (Sigma-Aldrich Chemical Co, St. Louis, MO) and 50 mg/ml of gentamicin], and finally transported to the laboratory in an incubator at 38.5°C (Ref #19180/2101, Minitube, Verona, USA).

The oocytes from each donor were cultured individually and underwent *in vitro* maturation, fertilization and embryo culture processes following the laboratory protocols. The semen used was from two bulls.

Statistical analysis.

Experiment 1. To determine the degree of relationship between the antral follicle population and the AMH concentrations in Brahman oocyte donors, and AMH concentration variation during estral cycle, correlation and repeated measurement analysis was performed using R project 3.6.3 software.

Experiment 2. For data analysis, all values were tested for normal distribution, using the Kolmogorov-Smirnov test. The data were subjected to descriptive statistics and expressed as mean ± standard error of the mean (SEM) except for correlation. Serum AMH concentrations from 100 Brahman oocyte donors were classified into 3 groups as low, intermediate, and high AMH. Statistical analysis for AMH concentration, number of follicles and number of blastocysts were performed by ANOVA, followed by the Kruskall-Wallis test, with a significance level of 0.001. The data were analyzed with the R project 3.6.3 software.

RESULTS

Experiment 1. In the present study, it was possible to determine that there were no significant differences in circulating AMH concentrations on the different days of the estrous cycle in Brahman breed cows (Table 1). The immunoenzymatic technique was validated, results indicated an intra-assay coefficient of variation (CV) of 6.36%, and an inter-assay CV of 8.31%. In the donors evaluated during experiment 1 the mean AMH concentration was 0.580 ± 0.05 ng/ml. The mean follicular count was 22.27±8.18, and the correlation between AMH concentration and the follicular count was high (r=0.82; p<0.001).

Table 1. AMH concentration of Brahman oocyte donors at different days of estrous synchronization (day 8, 13 and 23 in relation to the day of device insertion – d0).

Day of sampling	AMH concentration (ng/ml)	Standard deviation	p value
8	0.625	0.070	0.632
13	0.529	0.070	
23	0.581	0.070	

Experiment 2. Serum AMH concentrations from the greater Brahman oocyte donor's subset, ranged from 0.02 to 2.69 ng/ml. The mean (± SD) and median AMH concentrations were 1.12 ± 0.44 and 1.06 ng/ml, respectively. Cows classified as low AMH, comprising more than 20% of the samples, had a mean of 0.31 ng/ml and a range of 0.02 to 0.67 ng/ml; donors classified as intermediate AMH had a mean of 0.93 ng/ml and ranged between 0.74 and 1.48 ng/ml; and those classified as high AMH comprised more than 20% of the samples, mean 1.85 ng/ml and ranged from 1.48 to 2.69 ng/ml (Table 2), there were significant differences between the ranges and mean values (p<0.001).

Table 2. Categorization of AMH concentration and total follicles count in Brahman oocyte donor cows.

AMH categorization (mean concentration in ng/ml)	Total follicles count (± SD)	p value
High (1.85)	32.80 ± 8.94[a]	0.0001
Intermediate (0.93)	21.20 ± 4.30[b]	
Low (0.31)	18.20 ± 3.30[c]	

The correlation between AMH concentration and number of follicles was moderately high (0.73) and significant (p<0.0001) as presented in Figure 1.

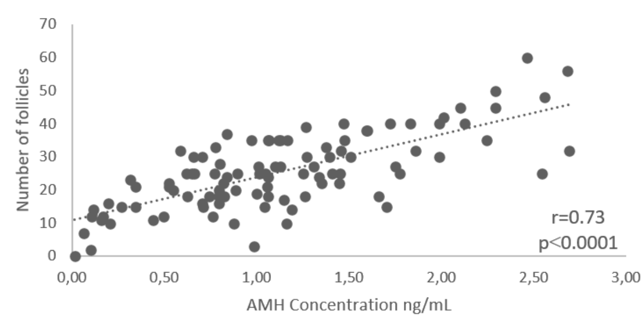

Figure 1. Correlation between AMH and number of follicles in Brahman oocyte donor cows.

It was observed that as the *in vitro* process progressed, the correlation between AMH decreased in relation to the number of recovered oocytes (0.68), oocytes that entered to the maturation process (0.65), oocytes that entered to the fertilization process (0.60), cleaved embryos (0.56) and blastocysts (0.54; Figure 2), however, theses correlations are considerate moderate ($p < 0.001$).

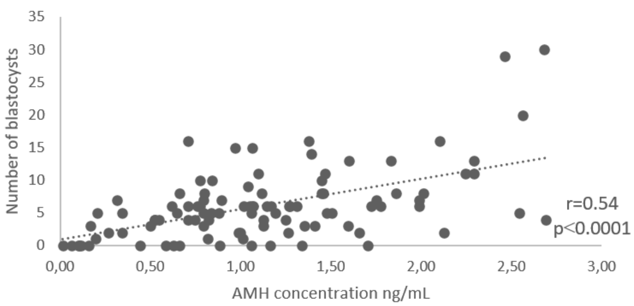

Figure 2. Relationship between AMH concentrations and the number of blastocysts from Brahman oocyte donor cows.

DISCUSSION

To achieve productive efficiency in systems, it is essential to look for selection parameters that are measurable, repeatable, and that depend as little as possible on individual appreciation in order to reduce subjectivity in the selection parameters of animals that will be used as breeders and provide relevant characteristics to the herd, allowing the production system to be more competitive. In this sense, the use of AMH as a selection tool for donor females can help in the detection of animals with greater potential as oocyte donors for the application of reproductive biotechnologies.

Validation of the ELISA AMH blood detection technique for Brahman donors. The obtained intra- and inter-assay coefficients of variation were low enough to allow the use of the ELISA technique for bovine AMH as a reliable tool, through which repeatable results can be obtained and therefore be used as a selection tool of females of the Brahman breed that have a greater ovarian reserve.

Previous studies, with other cattle breeds have reported similar behavior to those reported in this study for the Brahman breed. For Holstein x Normando, AMH concentrations were reported between the range of 1.74 and 23.68 ng/ml, showing an intra-assay coefficient of variation between 3.4 and 11.3%, respectively (35). As well as concentrations between 0.033 and 0.125 ng/ml, for Holstein, with an inter-assay coefficient of variation between 3.6 and 11.8% (25).

Relationship between AMH concentration and antral follicle population. The number of antral follicles is an important characteristic in reproductive biotechnology procedures, since it is an indicator of the potential for *in vitro* embryo production. Our results indicate a strong correlation (r=0.73) between the number of follicles and AMH concentrations in Brahman oocytes donors. This association may be due to the fact that AMH is secreted by granulosa cells in small antral follicles, being similar to previous literature reports (8,9,10).

Studies involving measurements of circulating AMH concentrations have been carried out in several cattle breeds and some other production species, finding, for example, in Tabapuã cows, a Brazilian *Bos indicus* breed for meat production, presented mean values of 1.60 ng/ml, with ranges from 0.014 to 4.516 ng/ml (36) and a positive correlation between concentration of circulating AMH and the number of antral follicles. Those animals considered the most outstanding reproductively, based on different parameters of their reproductive history and those that at the time of the study, had a greater antral follicle count, presented mean concentrations of 1.15 ng/ml, the animals with an intermediate follicular population, oocyte production had a mean concentration of 0.73 ng/ml, and those individuals who historically presented lower production had a mean concentration of 0.44 ng/ml (36).

Another study, carried out comparing AMH concentrations in Holstein and Gyr heifers, subjected to a synchronization protocol, and collected samples on the day of ovulation, found that the mean concentrations of AHM homorne for *Bos indicus* (Gyr; 0.60 ± 0.09 ng/ml) were greater than for *Bos taurus* (Holstein; 0.24 ± 0.08 ng/mL) (19). Additionally, the present investigation demonstrated that among the *Bos indicus* breeds, Brahman presented a lower AMH concentration compared to Tabapuã and greater than those reported to Nelore and Gyr breeds.

For Angus breed, a mean AMH concentration of 0.070 ng/ml was found, for Charolais it was 0.041 ng/ml, for Holstein 0.028 ng/ml, while for Jersey breed 0.046 ng/ml, during the heat synchronization and natural estrous cycle (22), which demonstrated to be much lower values than we found for Brahman cows. According to another study, the mean AMH concentration found in female Holstein cattle was 0.368 ng/ml, ranging from 0.091 to 1.391 ng/ml (26).

The results of this research provide evidence that there is a positive correlation between the serum concentration of AMH and the number of antral follicles in Brahman oocytes donor. These results suggest that AMH could be a possible long-term endocrine marker of ovarian activity similar to the findings reported by Ireland et al (33), who infer that a single blood sample taken at a random stage of the estrous cycle to measure AMH concentration could be considered a reliable physiological marker to predict the relative number of follicles, contributing to the selection of cows with a higher potential for successful results in reproductive biotechnologies such as OPU and IVEP. In addition, to our knowledge, this work is the first study that reports the circulating AMH concentrations on different days of the estrous cycle and its relationship with ovarian reserve in Brahman donors for *in vitro* embryo production, which provides new information on the reproductive physiology of this breed.

Relationship between AMH concentration and embryos production In this study, a moderate positive correlation was found between blood AMH concentrations and the number of embryos, which agree with those obtained in the experimental work carried out by Monniaux et al (14), who observed that there was a positive correlation between the AMH concentration of donor cows and the number of obtained embryos. Cows with plasma AMH concentrations between 0.10 to 0.20 ng/ml and greater than 0.20 ng/ml produced a greater number of transferable embryos than cows with AMH concentration lower than 0.01 ng/ml.

The results of this study are also similar to those of Batista et al (20), in which a positive correlation was observed between the plasma concentration and the number of blastocysts produced from of the Nelore (r=0.62) and Holstein (r= 0.58) donor calves.

In another study by Guerreiro et al (28), donors classified with high AMH levels produced a significantly higher number of embryos compared to those with low AMH levels.

The concentration of circulating AMH, can be used as an indicator of ovarian reserve, with the advantage of remaining constant in all phases of the estrous cycle without being affected by synchronization protocols carried out on females (20,35), which was confirmed in the present study for Brahman cows.

Our results also partially coincide with those obtained in the experimental work carried out by Batista et al (20), in which donor cows were assigned to different groups based on the AMH concentration, obtained independently of the phase of the estrous cycle of the donor cows. These authors also observed that there was a positive correlation between the AMH concentration of donor cows and the number of antral follicles per cow.

The decrease in AMH concentration over time is expected, since the ovarian reserve reduces as the female presents more estrous cycles, due to a reduction in smaller number of follicles, as this hormone is secreted by the granulosa cells of small-sized antral follicles (21).

A positive correlation has been established between the number of oocytes obtained in a superovulation protocol and their subsequent aspiration and plasma levels of AMH. Vernunft et al (26), concluded that the technique allows the identification of groups of cows with the potential to be good or bad oocyte donors (indicating a potential tool to select better oocyte donors). Our results indicates that, although AMH can be an indicator of the number of oocytes, it cannot be considered an indicator of quality or viability of the embryos that may arise from their *in vitro* fertilization. These achievements could translate into protocols in which more oocytes could be obtained by aspiration and thus increase the success rate of *in vitro* embryo production, reducing generational intervals, optimizing the use of genetic resources considered valuable for their productive potential and reproductive capacity, positively influencing fertility in productive systems dedicated to the commercialize genetic material.

Correlations have been observed between high AMH concentrations and some productive parameters in cows. The relationship between circulating AMH concentrations and productive longevity in Holstein cows was investigated.

However, according to the authors, it was not possible to find a correlation between these two parameters, but they observed that those animals that had higher AMH concentrations, historically had a better and outstanding reproductive performance (37).

Circulating AMH concentration is useful in the identification of animals that are likely to have a better response to a treatment with gonadotropins when performing superovulation protocols or OPU sessions, being those cows with higher concentrations, the best oocyte donors, and the best candidates to participate in IVEP programs (27). In the future, with complementary research applied in the field, it can be possible to select animals using their circulating levels of AMH, since they would be expected to have a higher reproductive potential, and even those cows with higher levels of AMH circulating to be longer-lived and productive.

In conclusion, our data demonstrate that AMH is a feasible biomarker to indicate ovary reserve and *in vitro* embryo production efficiency in Brahman donors. To the extent of authors knowledge, this is the first study reporting the correlation between the serum AMH concentration on different days of the estrous cycle and ovarian reserve in Brahman oocytes donors, which provides new insights on reproductive physiology and response to reproductive biotechnologies in this breed.

Credit authorship contribution statement

Diego A Riveros Pinilla: investigation, methodology, writing - original draft, writing - review & editing; Carolina Bespalhok Jacometo: investigation, methodology, formal analysis, writing - review & editing, funding acquisition; Juan David Corrales Álvarez: methodology, formal analysis, writing - review & editing, funding acquisition; Liliana Chacón Jaramillo: investigation, methodology, formal analysis, writing - original draft, writing - review & editing, funding acquisition; Julio C Olaya Oyuela: writing - review & editing, funding acquisition.

Declaration of competing interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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