PLASMA ANTI-MÜLLERIAN HORMONE LEVEL AND ITS RELATIONSHIP TO OVARIAN RESERVE, SUPEROVULATION RESPONSE, AND EMBRYO SURVIVAL IN BRAHMAN (*Bos indicus*) FEMALES

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ABSTRACT

Indicators of superovulation response is needed for efficient in vivo embryo production and facilitate production of genetically superior animals. Relationships of plasma Anti-Mullerian Hormone (AMH) levels to ovarian reserve, superovulation response, and embryo survival in Brahman females were examined on January 2019 in Manabí, Ecuador. Twenty-one Brahman females were classified according to plasma AMH levels arranged in a completely randomized design with factorial treatment. Factor A corresponded to animal classification (cows and heifers), and factor B corresponded to AMH levels (100<200, 200<300, and 300<450 ρg/mL). Animals were treated with progesterone implant, estrogen benzoate injection, and intramuscular progesterone on Day 0 and a decreasing concentration of gonadotrophin on Day 4-6. Prostaglandin injection and implant removal were done on Day 6. On Day 8, gonadorelin was given and artificial insemination performed. Ovarian reserve and superovulation response were assessed by endorectal ultrasonography. Embryos were collected, quality evaluated, transferred, and pregnancy determined. Positive correlations (R^2:0.73) between plasma AMH and number of ovarian reserves and number of transferable embryos were observed with increasing rates of 6.28% and 3.54%, respectively. Heifers had significantly higher corpora lutea than cows (13.7 *vs* 8.81, P<0.05). Embryo survival was highest in animals with higher AMH levels suggesting that selection of donors in superovulation programs should consider young animals with AMH levels above 300 ρg/mL for higher efficiency.

Key words: Follicle, corpora lutea, artificial insemination, MOET, embryos

INTRODUCTION

Anti-Müllerian hormone (AMH), also known as Müllerian Inhibiting Substance (MIS), is a homodimeric disulfide-linked glycoprotein that belongs to the transforming growth factor-β superfamily with a molecular weight of 140 kDa (Josso et al. 2001) corresponding to 553-575 amino acids and expressed only in gonads (Cate et al. 1986). It is expressed early in gonadal differentiation of the male before seminiferous tubules become morphologically distinct with the testis, causing the regression of Müllerian duct which in the normal female embryo develops into the reproductive tract

(Cate et al. 1986) thus, it is also called as MIS. In cycling females, it is produced by the granulosa cells of early antral follicles on the ovary (Monniaux et al. 2008; Rico et al. 2011) and is used as a marker for the ovarian reserve (Ireland et al. 2007 and 2008; Rico et al. 2009). The secretion of AMH is greatest in 2 to 5 mm follicles (Van Rooij et al. 2002) while the expression of AMH decreases as the follicles grow and enlarge, with expression essentially lost when the follicles reach 8 mm diameter or larger (Weenen et al. 2004). With the advances in reproductive biotechnologies concerning embryo production, understanding the role of AMH in the ovarian status of females is important, especially in superovulation treatment for *in vivo* embryo production. While effective *in vitro* embryo production results were achieved in cattle (Boni 2012; Morotti et al. 2014; Monteiro et al. 2017), improvement of the success rate of *in vivo* embryo production remains a challenge to decrease the cost of production and enhance efficiency and profitability. In *in vivo* embryo production, the goal of superovulation is to stimulate several small antral follicles to grow and mature, resulting in multiple ovulations. Therefore, the set of small antral follicles available for stimulation is of great importance in superovulation treatments.

Follicular counts and AMH levels in circulation represent valuable tools for predicting superovulation response due to its high correlation with ovulation and embryo recovery (Center et al. 2018). Similarly, cows with a large number of follicles had more recoverable and transferable embryos (Ireland et al. 2011).

Several studies on dairy cattle reported a strong positive correlation between circulating AMH levels and *in vivo* embryo production after superovulation, demonstrating that those animals with higher AMH levels had the best superovulation response that resulted in a greater number of corpora lutea, total embryo produced, and total transferable embryos (Monniaux et al. 2010; Rico et al. 2012; Souza et al. 2015; Aziz et al. 2017; Hirayama et al. 2017). The correlation was found in plasma AMH levels and ovarian follicle count after superstimulation treatment, the number of corpora lutea after superovulation (Rico et al. 2012; Rico et al. 2009; Souza et al. 2015), and the number of embryos produced in primiparous and multiparous cows (Souza et al. 2015). Circulating AMH levels were positively correlated with fertility by artificial insemination and natural service after the detection of spontaneous estrus in dairy cows (Ribeiro et al. 2014). Therefore, the evaluation of circulating AMH levels could help in increasing breeding efficiency and reproductive performance through the selection of animals for embryo production and insemination.

It should be noted that there are divergent results regarding the effects of AMH on superovulation response, which has been associated with the differential behavior of different cattle breeds. There is no relationship between AMH levels and *in vitro* embryo production rates in Holstein and Nelore females (Guerreiro et al. 2014), as well as young Nelore females (Zacarias et al. 2018). In the case of Japanese black cows, repeated superovulation sessions reduced the accuracy in predicting ovarian response by measuring AMH concentration because the ovarian response and plasma AMH concentration change progressively (Hirayama et al. 2017). These support the claim that superovulation can alter follicular development, oocyte maturation, ovulation, and sperm transport, which can ultimately affect normal fertilization and embryo development, resulting in a greater number of unfertilized oocytes and poor-quality embryos (Kafi and McGowan 1997).

Given the discrepancy in superovulation response in different bovine breeds, this study sought to evaluate the relationship between plasma AMH levels and ovulatory response in Brahman (*Bos indicus*) females.

MATERIALS AND METHODS

Location and animals. This study was carried out on January 2019 in the stable of the Aura Germania Company, located in the municipality of Paján, province of Manabí, Ecuador, at the coordinates LS 01°

33’ 0” and LO 80° 25’ 60”, at an altitude of 110 masl, with an average annual temperature of 24º C, and average annual rainfall of 1500 mm which has the necessary facilities for animal handling, including embryo collection room and an insemination and embryo transfer chute, where animals receive the same management practices. The study utilized 21 Brahman females at the herd of the Aura Germania Company, selected based on the uniformity of age (1-4 yrs), parity (1), and body condition of 3-4 where 1 is emaciated and 5 is extremely fat. The animals were kept in paddocks, fed mainly on *Cynodon nlemfuensis* and *Panicum maximum* (Saboya) grass, and supplemented with concentrated minerals to meet nutritional requirements. Water is supplied *ad libitum* from natural sources. The analyses of the levels of plasma AMH were conducted in the Biotechnological Laboratory of the Polytechnical Agricultural College of Manabí Manuel Félix López (ESPAM-MFL), located in the municipality of Bolívar, province of Manabí, Ecuador at the coordinates, LS 0° 49’ 23” and LO 80° 11’ 01”, and an altitude of 15 masl.

This study was carried out under the Animal Welfare Act Regulations of ESPAM MFL guidelines with approval granted by the Animal Ethics Committee of the National Institute of Agricultural Research of Ecuador with reference number ESPAM-MFL 830034001.

Experimental design. Cows and heifers selected based on the uniformity of age, parity, and body condition were subjected to blood sampling and subsequent determination of plasma anti-Müllerian hormone (AMH) levels in the blood. Three analysis groups were formed; the first group were animals with AMH levels ranging from 100<200 ρg/mL; the second group were animals with 200<300 ρg/mL AMH levels, and the third group were animals with 300<450 ρg/mL AMH levels. The relationship between AMH levels and the ovarian reserve which is the number and size of ovarian structures before and after superovulation treatment, the number of fertilized oocytes, and number of collected embryos after artificial insemination and *in vivo* collection, and the pregnancy rate after embryo transfer were examined.

Blood sample collection and hormone assay. Four mL of blood samples were collected three times at weekly interval before superovulation treatment from each animal. This was done by a puncture in the jugular vein using a gauge 16 x 1.5 disposable needle and blood was drawn by vacuum into a BD Vacutainer® NH tubes (Becton Dickinson and Co.) with sodium heparin. The blood samples were centrifuged immediately at 3,200 g for 10 minutes at 4 °C and instantly plasma was collected and stored at -20 °C until the ELISA test was performed.

For AMH determination, ELISA test, MOFA® "KITS" (AMH ELISA simple test Kit Bovine Serum, 40 samples Max / Kit 21700/100) was used for processing the samples. Plasma samples were thawed in a water bath at room temperature, briefly shaken on a "vortex" and centrifuged at 3,200 rpm for 10 minutes at 4 °C, then incubated for 12 hours at 4 °C in the presence of a first antibody, and for 1.5 hours at room temperature in the presence of a second antibody. A 50 µL of the sample was taken and AMH levels was read using the "Infinite 200 PRO Multimode Multiplate Reader" following the methods described by Rico et al. (2012).

Superovulation treatment. Heifers and cows were treated differently in terms of the concentration of Folltropin (Folltropin-V, Bionichi Animal Health Canada, Inc) and the schedule of the first dose of artificial insemination (AI). On Day 0, all donors received 0.5 g of controlled-release natural progesterone implant in a silicone device (DIB® 0.5, Zoetis, Madrid) and injected intramuscularly with 2.5 mg Estradiol Benzoate (Gonadiol, Zoetis, Madrid, Spain) and 50 mg P4 (Gestavec® 25, Vecol, Bogota, Colombia) at 6:00 h. On Day 4 to 7, Folltropin was administered in a decreasing manner with heifers given 30, 20, 20, 10 mg while the cows given 50, 40, 20, 10 mg in the morning and in the afternoon at 6:00 and 18:00 h, respectively. Each 20 mL vial of Folltropin contains FSH equivalent to 400 mg NIH-FSH-P1, diluted in a 20 mL vial of USP Bacteriostatic Sodium Chloride injection. On Day 6, PGF2 alpha (Cloprostenol base 250 ug/mL, Ciclase Dl, Zoetis, Madrid, Spain) was administered

to all animals and the progesterone implant was removed. On Day 8, heifers were artificially inseminated at 6:00 h and 100 µg gonadorelin (Pig pituitary GnRH, Gonadorelin 0.005 g, Ceva Sante Animale, France) was injected at the 18:00 h while the cows were injected with 100 µg gonadorelin on 6:00 h followed by AI on the 18:00 h. On Day 9, heifers and cows were artificially inseminated at 6:00 h for the 2nd dose. On Day 15, the response to superovulation treatment was examined at 6:00 h.

Assessment of reproductive parameters and responses. To assess the superovulation responses of the treated animals, the number and size of follicles categorized as small (<4mm), medium (4-7mm), and large (>7mm) on their left and right ovaries were determined through endorectal ultrasonography attached with 7.5 MHz linear probe (Mindray Dp50 vet) at the time of artificial insemination. Additionally, the number of corpora lutea at the time of embryo collection before catheterization was determined by ultrasonography of the superovulated animals.

Embryo collection. Embryo collection was performed through transcervical intrauterine catheterization and uterine lavage using a Foley catheter 16 FG according to the methodology described by Vivanco (2002) and embryo quality was evaluated according to the IETS (Stringfellow and Seidel 2000) classification, which distinguishes the number of fertilized, degenerated and unfertilized embryos. The *in vivo* survival rates in transferred embryos were recorded to provide a reference on the pregnancy rate after embryo transfer of embryos derived from Brahman females with different levels of plasma AMH.

Recipient preparation. All collected embryos with transferable quality and appropriate stage (compact morula, blastula, expanded blastula, hatched blastula) were transferred fresh to recipient animals within the same stable on the 7th day from the beginning of the estrous cycle (Day 0-estrus), and on synchrony with the donor. The transfers were made by non-surgical procedures as described by Hufana-Duran et al. (2004).

Data analysis. The experiment consisted of a total of 21 animals. Descriptive statistics were used to analyze the variable number of embryonic structures such as the number of fertilized, degenerated and unfertilized oocytes; and the ovarian structures which are composed of the number of corpora lutea and anovulatory follicles; and the embryo recovery rates in Brahman females.

For the analysis of variance, a completely randomized experimental design with a factorial arrangement was used. Factor A corresponded to animal category (cows and heifers) and factor B corresponded to the AMH levels (100<200 ρg/mL, 200<300 ρg/mL and 300<450 ρg/mL) established from the blood samples. Each animal represented an experimental unit, and at least three repetitions were made for each treatment combination. The initial values of the quantified variables were previously examined through the Shapiro-Wilks test for normality and Bartlett's Test for Homogeneity of Variances. In the cases where the ANOVA assumptions were not met, the data were transformed for verification. The means were compared through the Tukey test at a 5% probability.

The degree of functional relationship among the quantified variables was determined through correlation and regression analyses. In the regressions, the number of ovarian structures was adjusted with the AMH ranges and transferable embryos. In Pearson´s correlation analysis, for the variables on embryonic qualities, transferable embryos, degenerated embryos, unfertilized embryos, corpora lutea, anovulatory follicles, ovarian structures and embryo recovery, the significance was determined through the Student's t-test at 5 and 1% probability. The statistical analyses were performed with the InfoStat® software (Di Rienzo et al. 2017).

RESULTS AND DISCUSSION

A total of twelve (12) heifers and nine (9) cows of single parity were used in the study according to their AMH level in blood plasma. Table 1 presents the profile of the animals used in this study.

Table 1. Category and number of animals used in the study.

AMH Level, ρg/mL	Heifer, n	Cow, n	Total
100<200	1	4	5
200<300	6	3	9
300<450	5	2	7
Total	12	9	21

AMH concentration and follicle number before superovulation. Comparison of the mean number of ovarian follicles in Brahman heifers and cow before superovulation treatment is presented in Table 2. Between heifers and cows within the same AMH level, no statistical difference was observed. A difference was observed among animal category where heifers had higher reserve of small follicles than cows; 37.17±1.70 vs. 34.22±1.70 ($p<0.05$), respectively. On AMH level, cows and heifers with 300<450 ρg/mL AMH had a significantly ($p<0.05$) higher number of small and medium size follicles, 47.08±3.33 and 46.00±4.71 against 30.05±2.72 and 37.00±4.36 and 25.54±3.48 and 28.50±4.71 follicles in 200<300 and 100<200 ρg/mL AMH groups, respectively.

The ovarian structures of the Brahman females with different levels of plasma AMH is presented in (Fig. 1). The total number of follicles was significantly ($p<0.05$) higher (46.54) in 300<450 ρg/mL AMH group than in 200<300 (33.52) and 100<200 (27.0) ρg/mL AMH groups.

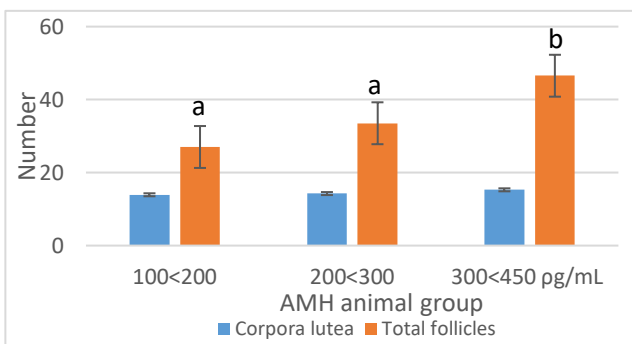

Fig. 1. Ovarian structures of Brahman females with different AMH plasma concentrations before superovulation treatment. Orange bars with different superscripts are different at $p<0.05$.

The higher number of ovarian follicles in Brahman females with >300 ρg/mL than those with <300 ρg/mL AMH plasma concentration indicates that the population of follicles contributes significantly to plasma AMH concentration. AMH is specifically expressed by ovarian granulosa cells in mammals (Vigier et al. 1984; Takahashi et al. 1986; Monniaux et al. 2008) making it a reliable endocrine marker of ovarian follicular population (Ireland et al. 2008; Rico et al. 2009, 2012; Monniaux et al. 2010; Souza et al. 2015). The number of small antral follicles is the direct target of ovarian stimulation treatments (Cardoso et al. 2018) and based on the results of this research, the plasma level of AMH is a good indicator in selecting Brahman females for superovulation treatment.

Table 2. Follicular reserve of Brahman heifers and cows before superovulation treatment.

Ovarian structures, M±SEM	Animal group according to AMH plasma concentration in ρg/mL							
	100<200		200<300		300<450		Total	
	Heifer	Cow	Heifer	Cow	Heifer	Cow	Heifer	Cow
Small follicle	16.33±3.45^{a}	12.63±2.55^{a}	22.85±3.19^{abc}	17.72±1.99^{ab}	30.0±3.45^{c}	28.75±2.44^{cb}	37.17±1.70^{A}	34.22±1.70^{B}
Medium follicle	12.16±1.95^{a}	12.33±1.13^{a}	14.14±1.81^{a}	13.00±1.44^{a}	16.00±1.95^{a}	18.33±1.38^{a}	14.10±1.91^{A}	14.55±1.31^{A}
Total follicles	28.50±4.71^{a}	25.54±3.48^{a}	37.00±4.36^{abc}	30.05±2.72^{ab}	46.00±4.71^{bc}	47.08±3.33^{c}	37.60±4.59^{A}	34.22±3.17^{A}

Means with different small letter superscript in same row are significantly different ($p<0.05$).
Means with different capital superscript (total heifer/cow) within row differs significantly ($p<0.05$).

Table 3. Analysis of variance for variables corpora lutea, anovulatory follicles, ovarian structures, transferable, degenerated, unfertilized oocytes, and embryo recovery rate in Brahman females.

Sources of variation	DF	Mean Squares						
		Corpora lutea†	Anovulatory follicles †	Ovarian structures †	Embryos†			Embryo recovery rates††
					Transferable	Degenerated	Unfertilized	
Animal categories	1	2.457*	1.099	0.558	0.492	0.159	3.524	29.304
AMH range	2	0.766	1.711	2.010**	4.417**	0.062	0.655	1160.026
Animal categories* AMH range	2	0.384	0.581	0.058	0.302	0.352	0.143	543.859
Error	15	0.571	0.732	0.312	0.192	1.020	1.258	691.857

† = Transformed data $(X+1)^{-0,5}$; †† = Transformed data arcsine(X); significant at $5^{(*)}$ and $1^{(**)}$ % probability for F test.

The higher follicular reserve in animals with >300 ρg/mL AMH plasma indicated that AMH concentrations have a high degree of relationship with the antral follicle count and these characteristics are good indicators in selecting animals for superovulation treatment as observed in Holstein cows (Benyei et al. 2003; Mossa et al. 2012).

AMH concentration and superovulation response. Analysis of variance for variables corpora lutea, anovulatory follicles, ovarian structures, embryos such as the transferable, degenerated, and unfertilized oocytes, and embryo recovery rate in Brahman females is presented in Table 3. Results showed a significant difference between animal categories i.e. heifer *vs.* cow, in the number of corpora lutea ($F=2.457$, $p<0.05$). In the AMH groups 100<200 ρg/mL, 200<300 ρg/mL and 300<450 ρg/mL, a significant difference was observed in the ovarian structures ($F=2.010$, $p<0.01$) and in the number of transferable embryos ($F=4.417$, $P<0.01$). No significant differences were found on the interaction between animal category and AMH range for the evaluated variables suggesting that in this study, the number of corpora lutea is the distinct indicator on the difference between heifer and cows and the ovarian structures and number of transferable embryos are the distinct indicators on the difference of the AMH level.

The number of superovulation response and quality of recovered embryos after superovulation treatment among AMH concentration groups are presented in Table 4. In these parameters, Brahman females in 300<450 ρg/mL AMH group had the significantly higher ($p<0.05$) number of ovarian structures i.e. anovulatory follicle and corpus luteum than the 100<200 AMH groups; 21.17 *vs* 10.67 ovarian structures. Transferable embryos were also highest in 300<450 ρg/mL AMH group than the other groups; 7.67 *vs*. 2.25 and 1.00 transferable embryos, respectively (Table 4). Similarly, a significant difference ($p<0.05$) was also observed on the embryo recovery rate between AMH groups though no difference was observed on the number of recovered degenerated and unfertilized oocytes. A significantly higher ($p<0.05$) number of corpora lutea was also observed after superovulation treatment in 300<450 AMH and 200<300 AMH groups (Fig. 2) suggesting that Brahman females with high AMH plasma concentration are best animals for superovulation that supports the studies conducted in other bovine species (Silva-Santos et al. 2014; Baruselli et al. 2015; Ghanem et al. 2016). Moreover, the number of the ovarian reserve is associated with the quality of oocytes (Ireland et al. 2009), embryonic competence (Tessaro et al. 2011), and fertility (Mossa et al. 2012; Ribeiro et al. 2014; Jimenez-Krassel et al. 2015).

Table 4. Superovulation response and quality of recovered embryos after superovulation of Brahman females with different class of AMH plasma concentration.

Parameters, Mean±SEM	**100<200**	**200<300**	**300>450 ρg/mL**
Ovarian structures*	10.67±0.32[a]	18.88±0.27[b]	21.17±0.32[b]
Transferable	1.00±0.19[a]	2.25±0.16[a]	7.67±0.19[b]
Degenerated	1.36±0.43[a]	1.35±0.36[a]	1.54±0.43[a]
Unfertilized	2.17±0.48[a]	2.11±0.41[a]	1.55±0.48[a]
Recovery rate, %	53.18±11.39[a]	61.01±9.60[ab]	81.53±11.39[b]

*Anovulatory follicles and corpus luteum.
Means with different superscripts within row differ significantly (Tukey $p<0.05$).

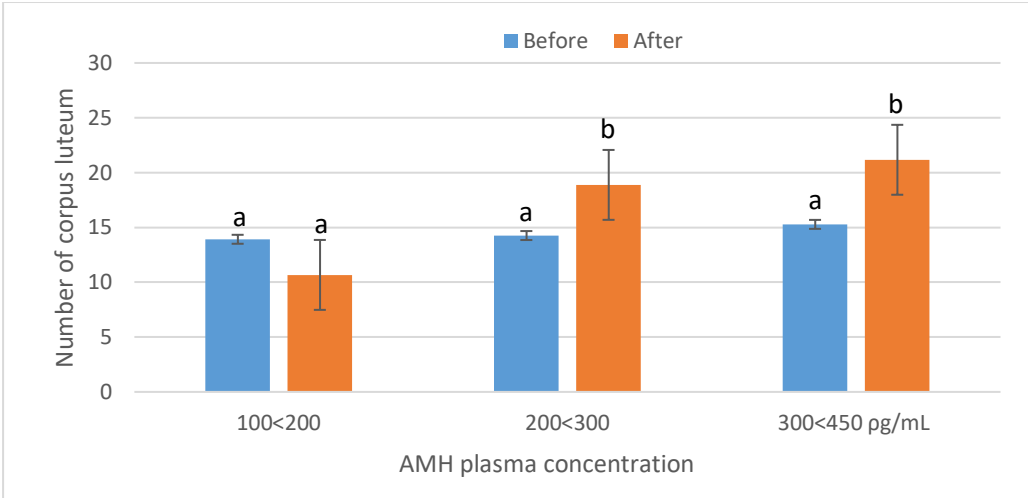

Fig. 2. Number of corpus luteum (CL) before and after superovulation treatment in Brahman females with different AMH plasma concentrations. Bars with different superscript letters within AMH plasma concentration are different at $p<0.05$.

The results after superovulation show that the high number of small follicular reserve have resulted in significantly higher number of follicles recruited to grow, mature, and ovulate resulting in the observed higher number of corpora lutea and embryos recovered in >300 ρg/mL AMH group. The number of follicles at the start of superovulation is positively correlated with the average number of corpora lutea and the number of collected embryos suggesting that the AMH level is a good indicator in the selection of Brahman females for superovulation. With the cost of superovulation treatment and the target of securing higher number of embryos for embryo transfer, selecting donor females with high efficiency is very important to optimize time and resources. With these results, it was deduced that examining the ovarian reserve and or checking the AMH level are important tools in the selection of donor animals in a multiple ovulation and embryo transfer program. These findings were also observed in beef cattle (Santos et al. 2016; Center et al. 2018) and in Nelore and Holstein breeds (Guerreiro et al. 2014). Though a high variation depending on the type of animal and high repeatability within individuals exist (Morotti et al. 2017), the observations that correlation of plasma AMH concentration on the number of collected and transferable embryos was weaker during the gestation period than during the periods prior to artificial and postpartum insemination in Japanese black cattle (Nabenishi et al. 2017); the concept that an antral follicle count is a useful tool for embryo production is reinforced by the impact on embryo production for genetic gain and reproductive performance. The plasma AMH concentrations before superovulation vary among animals but this correlates positively with the number of ovulations and transferable embryos produced which conforms with earlier reports in dairy cattle (Monniaux et al. 2010; Rico et al. 2009). With these, it can be deduced in this study that plasma level of AMH is a positive endocrine test in selecting Brahman females for superovulation treatment with checking the ovarian reserve as a physical indicator that a technician can perform in the absence of endocrine test and ultrasonography.

Animal category. The comparison of means by the Tukey test at 5% probability among the categories (Table 5) indicates that corpora lutea has the highest mean value (13.7±1.33) for heifers after superovulation, which were statistically higher ($P<0.05$) than those observed for cows (8.81±1.25). These results show that heifers had the higher number of <4 mm follicles than cows suggesting that the largest follicle population is present in young animals and gradually decreases as the animal ages. The decrease in follicular reserve starts when the animal reached puberty and undergo the regular estrous cycle where a follicle is recruited to grow, ovulated or reached terminal follicular growth and atresia (Monniaux et al. 2008; Rico et al. 2009; Macias-Andrade et al. 2020). The large follicular population

in heifers resulted in the significantly higher corpora lutea observed after superovulation treatment than cows (Table 4) suggesting that heifers are potential targets for superovulation treatment.

Table 5. Number of corpora lutea after superovulation in Brahman heifers and cows.

Category	Mean±SEM
Heifers	13.70±1.33[a]
Cows	8.81±1.25[b]

Means with different superscript are statistically different (Tukey p≤ 0.05).

In both heifers and cows, higher numbers of small follicles were distinct in >300 ρg/mL AMH group. These findings show that AMH concentrations represent an excellent endocrine marker of the number of small antral follicles that constitute the direct target of ovarian stimulation treatments in Brahman. This was also observed in cows of Normande and Holstein breeds (Monniaux et al. 2010). These results demonstrate that the antral follicle count and AMH plasma concentration are determining characteristic in bovine females and it has great influence on the efficiency of reproductive biotechnologies and reproductive performance. These findings provide a good reference in selecting the donor for a multiple ovulation and embryo transfer program for livestock genetic improvement.

The quantitative cause-effect relationships of the AMH levels are shown in Fig. 3. For the number of ovarian structures, a direct linear relationship was observed, represented by the equation $\hat{y}$=0.0628x-0.43 with a R^2=0.73 (Fig. 3A); while for the number of good quality embryos, a direct relationship was also found, represented by the equation $\hat{y}$=0.0354x-6.04 with a R^2=0.73 (Fig. 3B). It verified that as AMH levels increase, the number of ovarian structures increases by 6.28% and the number of good quality embryos increases by 3.54%.

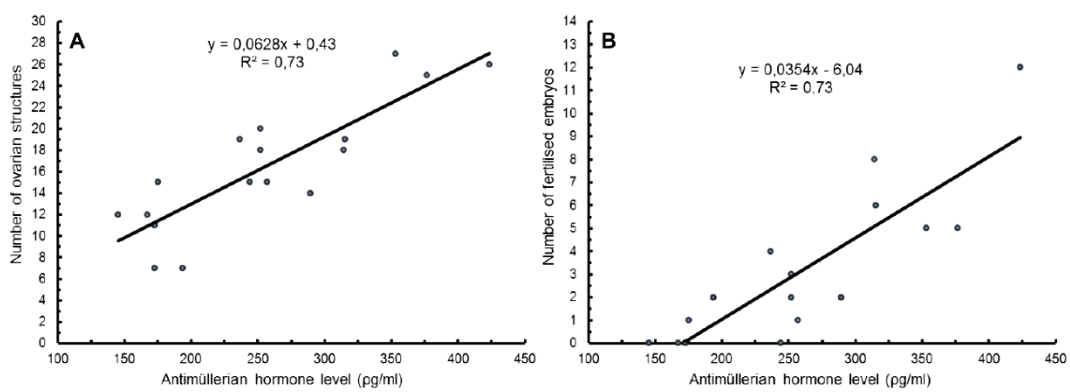

Fig. 3. The relationship among antimüllerian hormone levels, number of ovarian structures (A) and number of fertilized embryos (B) in Bos indicus bovine females.

Correlation coefficients of variables associated with superovulation response in *Bos indicus* Brahman heifers is presented in Table 6. Significant positive correlations were observed in embryonic structures and transferable embryos (r=0.459, p<0.05), degenerated embryos (r=0.559, p<0.01), unfertilized oocytes (r=0.478, p<0.05), corpora lutea (r=0.882, p<0.01), ovarian structures (r=0.656, p<0.01), and embryo recovery (r=0.693, p<0.01).

Table 6. Correlation coefficients of variables associated with superovulation response in *Bos indicus* heifers.

Variables	**Embryonic structures**	**Transferable embryos**	**Degenerated embryos**	**Unfertilized oocytes**	**Corpora lutea**	**Anovulatory follicles**	**Ovarian structures**	**Embryo recovery**
Embryonic structures	1	0.036	0.008	0.028	0.001	0.333	0.001	0.001
Transferable embryos	0.459*	1	0.438	0.267	0.087	0.911	0.128	0.034
Degenerated embryos	0.559**	0.179	1	0.252	0.021	0.761	0.060	0.211
Unfertilized oocytes	0.478*	-0.254	-0.262	1	0.051	0.377	0.290	0.159
Corpora lutea	0.882**	0.383	0.499*	0.431	1	0.091	0.002	0.088
Anovulatory follicles	-0.222	-0.026	-0.071	-0.203	-0.378	1	0.031	0.621
Ovarian structures	0.656**	0.343	0.417	0.242	0.639**	0.471*	1	0.036
Embryo recovery	0.693**	0.464*	0.285	0.319	0.381	0.115	0.459*	1

Correlation coefficient below the main diagonal. The probability associated with the test above the main diagonal.
Significant at 5(*) and 1(**) % probability for F test.

These results show that the number of embryonic structures and retrieval after superovulation depend on the ovarian structures present before superovulation treatment and this is associated with the number of corpora lutea present on the ovary. The quality of the retrieved embryos is classified as transferable, degenerated, and unfertilized and while all these parameters increased, the number of degenerated embryos showed higher correlation suggesting that as the number of embryonic structures increased after superovulation treatment, a high degree of degenerated embryos was also observed. The high incidence of degenerated embryos found in this study was not investigated but this could be due to aneuploidy (Hufana-Duran, 2009) as the embryos are of different stages of development. The number of degenerated embryos is also correlated with the number of corpora lutea ($r=499$, $p<0.05$) which means that these degenerated embryos are from ovulated oocytes fertilized by a sperm cell(s) that for unknown reason failed to develop to good quality and transferable embryos. The events involved in the embryo growth and survival are directly or indirectly related to cytokines, steroids, metabolites, and growth factors that when one of these compounds fails, it normally leads to the death of the embryo (Valadao et al., 2018). By triggering the ovaries to have more follicles to grow and develop during the superovulation treatment, limitations in these physiologic factors resulted in the high incidence of degenerated embryos and this is related to the high correlation of anovulatory follicles with ovarian structures ($r=0.471$, $p<0.05$). While superovulation treatment has resulted in higher ovarian structures; the mechanism for ovulation, normal oocyte maturation, and fertilization have to be taken into consideration to avoid anovulatory follicle and degenerating embryos. Considering these factors during the treatment might help improve the retrieval of transferable embryos.

The transferable embryos increased as embryo recovery increased ($r=464$, $p<0.05$) suggesting that the skill of the technician performing the *in vivo* collection of embryos is very important. The technician has to ensure that all the embryos present in the uterine horn are retrieved to increase the number of transferable embryos.

Embryo survival after embryo transfer. Out of the transferable embryos recovered from Brahman females, pregnancy and full-term development of calves were 0 (0/2), 50.0% (3/6), and 57.14% (4/7) in 100<200, 200<300, and >300 ρg/mL AMH groups, respectively (Table 7). While the number of transferred embryos were limited, these results show that the presence of AMH in the system is helpful in improving the development potential of the resultant embryo. It has to be noted that AMH is a key mediator in regulating steroidogenesis inhibiting estradiol secretion by reducing the expression of the aromatase enzyme CYP19 (Eilsø Nielsen et al. 2010) and in regulating progesterone production in granulosa cells *in vitro* (Yding Andersen et al. 2008). These observations suggest that the >300 ρg/mL AMH levels in the >300 AMH group is contributory in providing the developing embryos a conducive environment to grow and acquire the developmental competence needed resulting in higher pregnancy rate after embryo transfer.

Table 7. Pregnancy rate after transfer of embryos collected from the different AMH groups of Brahman heifers and cows.

Category	**Pregnancy rate (%) of AMH Group, ρg/mL**			
	100<200	**200<300**	**300<450**	**Total (Ave.)**
Heifer	0 (0/1)	66.67 (2/3)	50.00 (2/4)	50.00 (4/8)
Cow	0 (0/1)	33.33 (1/3)	66.67 (2/3)	42.86 (3/7)
Total/Ave.	0 (0/2)	50.0 (3/6)	57.14 (4/7)	46.67 (7/15)

The reduction of estradiol concentration and increase progesterone level during fertilization and early stage of development of the embryos in >300 AMH group are contributory to the higher

developmental competence among embryos retrieved from donors in the >300 AMH group. This claim is reinforced by the observation in this study where a higher number of corpus luteum is present in >300 AM group and has a significant relationship with the number of fertilized and transferrable embryos. These results suggest that the AMH concentration in the blood is a potential reference in selecting females with good superovulation response in Brahman females.

The results of this study demonstrated that circulating AMH has significant positive correlations with follicle count, ovulation rate, the number of recovered embryos, and embryo survival in Brahman females. These findings are consistent with earlier reports in beef cattle (Center et al. 2018), in *Bos indicus* (Nelore), and *Bos taurus* (Holstein) donors (Zangirolamo et al. 2018). With these results, the circulating AMH level is recommended as a tool in the selection of Brahman females for superovulation treatment to ensure more efficient and profitable application of the MOET activities. It can be used as an auxiliary tool for selecting donor cows for embryo production in multiple ovulation and embryo transfer program and can also be applied for oocytes retrieval in ovum pick up activities.

The positive correlation among plasma AMH levels and ovarian follicle count and corpora lutea after superovulation were also observed in Holstein cows (Rico et al. 2012; Rico et al. 2009) and in the number of embryos produced in primiparous and multiparous cows (Monniaux et al. 2010; Souza et al. 2015). These results suggest that AMH can be used as a predictive physiological marker for fertility in Brahman females. This claim is supported by earlier reports in Holstein cows undergoing an ovulation synchronization protocol, since cows with high AMH levels (> 300 ρg / mL) had a higher pregnancy rate after artificial insemination, required less services per conception, and had fewer day open compared to cows with normal levels (<300 ρg / mL) (Aviles et al. 2017). While it has been determined that there are differences in serum levels of *Bos indicus* and *Bos taurus* heifers (Batista et al. 2014) and even within breeds of the same species (Ribeiro et al. 2014), the results of this study demonstrated that more embryos are recovered from Brahman females with higher AMH levels, and therefore with highest follicle count.

Embryo production rate in cattle after superovulation is difficult to predict and varies between individuals (Monniaux et al. 2010). The results suggest that the selection of donors of female *Bos indicus* embryos should consider relatively young animals with plasma AMH levels above 300 ρg/mL. The blood measurement of AMH is of great value in determining the potential of a donor cow in producing transferable embryos.

CONCLUSION

The AMH concentrations are endocrine markers in selecting animals for superovulation and of the number of small antral follicles that are gonadotropin-responsive. The female Brahman (*Bos indicus*) with a high concentration of AMH (>300 ρg/mL) has a significantly higher follicular reserve, which after superovulation treatment, resulted in a higher number of corpora lutea and retrieved transferable embryos. These embryos possess higher developmental competence demonstrating the highest potential of animals with high AMH levels be used in superovulation treatment for *in vivo* embryo production. Heifers have a significantly higher superovulation response than cows. It is reasonable to emphasize the appropriateness of selecting relatively young *Bos indicus* females with plasma AMH levels greater than 300 ρg/mL, or cows from the upper quartile of the evaluated herd.

According to the literature and data cited above, AMH seems to be correlated with several fertility parameters, and it may be a tool that can contribute to the success of embryo production both *in vivo* and *in vitro*. However, there is a great need to study the real long-term impact of AMH on fertility, to establish specific parameters of AMH classification, and to understand the physiological causes of the variation in the AMH among individual female cattle.

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