Differences in plasma Anti Müllerian hormone levels and reproductive parameters between two bovine species: *Bos indicus* and *Bubalus bubalis*

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Abstract

The aim of this study was to evaluate the possible relationships between reproductive parameters and Anti Mullerian Hormone (AMH) levels in two closely related bovine species. AMH levels and the reproductive parameters of 50 water buffaloes (*Bubalus bubalis*) and 50 Zebu cattle (*Bos indicus*) of the same age and raised in similar conditions were evaluated. Clinical data were obtained from the farm in specialized designed format, and AMH was measured by ELISA. The results show that Zebu cattle had higher AMH levels than water buffaloes (P<0.0001), while water buffaloes exhibited better reproductive parameters: days open to pregnancy (P<0.0001), parity (P<0.0005), age at first calving (P<0.0001), and intercalving period (P<0.0001). Although both species have different reproductive parameters, no correlations were found between AMH and the reproductive parameters evaluated in either species. Finally, pregnant Zebu cattle and water buffalo showed a tendency to have lower AMH levels than non-pregnant counterparts. The results are paradoxical as the species with lower AMH levels exhibited better reproductive parameters, suggesting that the reproductive parameters within the herd do not depend solely on the ovarian reserve of females.

Key words: *Anti Müllerian hormone; water buffalo; Zebu cattle; reproductive parameters*

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Introduction

Water buffalo products are an emerging business that could contribute to strengthening and diversifying the economies of many countries, depending on their quality and diversity: meat, milk, and draught. At the global level, buffaloes contribute to 1.2% of the meat and 12.4% of the milk produced annually, with an increased rate of 12.4% in meat and 41.6% of milk (Singh and Balhara, 2016). Through technological advancements, it is possible to study different phenotypes that could be applied to improve production (FAO, 2016).

Ovarian reserve is defined as the number of primordial follicles that each female has for her reproductive life; this number is both limited and highly variable among both species and individuals. Antimullerian hormone (AMH) is a dimeric glycoprotein secreted by the granulosa cells of growing follicles (pre and early antral) within the ovary from the start of life to the end of the reproductive period. In women, AMH has been used as a tool to diagnose the state of the ovarian reserve, especially the transition to menopause (Broer et al., 2014), as a predictor of the response of follicular development to stimulation protocols in infertility treatments and pregnancy outcome. In animal production, AMH has been used as an endocrine marker to predict ovarian reserve, identify and determine the number of gonadotropin-responsive follicles in the cow (Rico et al., 2009), goat (Monniaux et al., 2011), and ewe (Lahoz et al., 2012). It has also been proposed as a clinical parameter for the selection of the most appropriated donors for superovulation programmes and the production of high numbers of transferable embryos in dairy cows (Rico et al., 2012). In mare oocyte aspiration programmes, AMH has been used to predict the number of follicles to be aspirated (Vernunft et al., 2013). Circulating concentrations of AMH and the antral follicle count (AFC) are positively associated, with reports that almost 25% of cows have a low AFC and relatively low concentrations of AMH (Jimenez-Krassel et al., 2015).

It has been reported that in *Bos taurus* dairy and beef heifers, a low AFC indicates small ovaries (Ireland et al., 2008; Cushman et al., 2009), lower probability of conception at the end of the breeding period (Mossa et al., 2012), reduced answer to ovary gonadotropin stimulation, lower number and quality embryos, lower circulating concentrations of progesterone and AMH (Jimenez-Krassel et al., 2015) and reduced endometrial thickness (Jimenez-Krassel et al., 2009), all parameters associated with poor reproductive performance. Females with a higher AFC are expected to have better reproductive performance, with a linear correlation demonstrated between AFCs and fertility in *Bos taurus* cattle (Evans et al., 2012). Since AFC has been positively correlated with AMH levels, AMH has also been proposed as a marker (Baldrighi et al., 2014). Cushman et al. (2009) reported a reduced pregnancy rate in beef heifers compared to high-AFC heifers, and similar reports have been made for milking animals (Mossa et al., 2012). Morotti et al. (2018) reported in Nelore females subjected to synchronization of ovulation that animals with lower AFCs had larger follicles and higher conception rates than groups with intermediate or greater AFCs, indicating that the marker is more indicative of specific stages of reproduction than of the animal's reproductive potential (Morotti et al., 2018).

For years, researchers have believed that the number of follicles and oocytes have an impact on fertility, and therefore it was considered that females with a better ovarian reserve could have better reproductive performance.

In animals there are few reports of the levels of AMH in reproductive and productive parameters, which some authors have suggested could be parameterized (Rico et al., 2012), and fewer reports complementing this research with the study of the physiology and pathogenesis of factors involved in the reproductive parameters. It is known that the observed parameters associated with reproductive performance are mainly affected by genetic and environmental factors, though there are few reports analysing biological markers and the outcome of the species (Hamel et al., 2010). Hamel et al. (2010) reported that biomarkers obtained in the granulosa cells of the human oocyte could be used as a pregnancy predictor in IVF programs, and therefore cattle and buffalo producers could use the same analysis from humans in combination with reproductive biotechnologies to select "normal working times for selection" for which females to use as replacements. This objective of this study was to evaluate the possible relationships between reproductive parameters and AMH levels of two closely related species of bovines: *Bos indicus* and *Bubalus bubalis.*

Materials and methods

The study was performed in November and December 2017 (breeding season), at two farms in the Middle Magdalena River zone, Colombia (6°18′48″N 73°57′00″W), classified as a humid tropical forest, elevation of 120 meters, average air temperature 24 °C, 79% humidity, and 2766 mm^3 annual rainfall. One hundred crossbred mature females, 50 buffaloes (*Bubalus bubalis*) and 50 cattle (*Bos indicus*) of proven fertility, with no anatomical abnormalities and similar weight (average ± standard deviation) 538±56.15 kg *vs.* 427±43.4 kg, and age 7.34±4.08 years *vs.* 7.16±3.6 years, were included in the study. Gestation state was diagnosed by palpation at the time of sampling. The data from the reproductive events for the three years preceding the beginning of this study were used to calculate parity, calving days, parity, first calving age, and calving days. Animals were managed under the same conditions, natural mounting, kept grazing at pasture (*Brachiaria humidicola*) and supplemented ad libitum with minerals. At the beginning of the study, blood samples were taken on the same day in an anticoagulated tube (EDTA, Vacutainer, Beckton Dikinson) with a 21 gauge needle for cattle and 18 gauge needle for buffaloes from the medial coccygeal vein and lateral caudal vein of the tail, respectively. Serum was aliquoted and frozen at -20 °C until AMH determinations using a commercial AMH ELISA kit (Cat No AL-114 Lot No 010616-B Ansh Labs, Webster, TX, USA). AMH levels were analysed individually and grouped into three different clusters: lowest 25th percentile, 25th to 75th percentile, and highest to 75th percentile.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) and range. Statistical differences were estimated using the Mann Whitney test and the correlation between variables were determinate by Spearman correlations using GraphPad Prism 7.0 Software (GraphPad Software, San Diego, CA, USA). Significant differences were considered at values of P<0.05.

Results

The results are summarized in Table 1. Water buffaloes had significantly lower AMH levels than cattle (Figure 1), better age at first calving and calving interval period and parity (P<0.0001). Despite the random selection of animals, it could be observed that calving days at sampling and parity were also significantly different (P<0.001).

No statistical differences in AMH levels between pregnant and non-pregnant animals were observed in either species. However, pregnant females of cattle and buffalo tended to have lower AMH levels than non-pregnant animals (1335±670.4 pg/mL *vs.* 1200±723.1 pg/mL, and 463.5±422.6 pg/mL *vs.* 322.7±295.1 pg/mL, respectively), reflecting the decrease in ovarian function during pregnancy. Also, there were no correlations between AMH and the reproductive parameters evaluated.

According to AMH levels, animals were grouped into three clusters: lowest 25th percentile (n=12 range: 184 - 697 pg/mL, 25th to 75th percentile (n=26 range: 706 - 1937 pg/mL, and highest to 75th percentile (n=12 range: 1958 - 2226 pg/mL), though no correlations were observed between reproductive parameters and AMH within the groups, and only the lowest percentile of cattle presented a significant correlation with parity (59%, P<0.05).

Discussion

Globally, there has been minimal genetic selection for fertility in water buffaloes, which is generally regarded as a poor breeder in Asian countries, with better fertility in Latin-American countries (Berdugo et al., 2013). In Latin America today, water buffaloes are a promising species and development of an efficient production model is underway. Currently, buffalo producers are applying models developed for cattle for the production of meat or milk, without considering the differences between the species. Additionally, water buffaloes have been reproduced without programmed genetic selection, though today there are significant challenges in management production systems and reproduction. Given the need to improve production, it is necessary to implement reproductive biotechnologies, such as artificial insemination and embryo transfer (Drost, 2007). To the best of our knowledge, this is first study in water buffaloes to evaluate possible correlations between reproductive parameters and AMH levels.

Reproductive parameters of the animals evaluated in this study are comparable with others reported for water buffaloes (Vergara et al., 2010) and cattle (Vergara et al., 2009). The results obtained here agree with other studies (Baldrighi et al., 2014) about the differences of AMH levels between cattle and water buffaloes. This is also the first report of AMH levels in Brahman cattle. There are high variations in AMH levels in Zebu cows and water buffaloes, though in animal production there are no reports of reference values for selection using this parameter, as there are for humans (La Marca et al., 2010). Hirayama et al. (2017) reported plasma AMH concentrations in Japanese Black cows ranging from 0.032 to 1.992 ng/mL, with concentrations, mean±SD and median of 0.334±0.318 ng/mL and 0.265 ng/mL, respectively. Ghanem et al. (2016) reported AMH concentrations ranging from 0.08 to 0.84 ng/mL in 19 samples of cow plasma.

As mentioned above, due to the high variation in cattle breeds, it is difficult to establish numerical values to define normality for comparison. As a consequence, in papers reporting AMH values, researchers compare and analyse their own studied population, grouping them by the levels obtained and analysing the data, evaluating whether there are relationships between the AMH levels and the examined parameters. Evans et al. (2012) evaluated the relationships of AFC, the number of oocytes, AMH concentrations, ovary size and function and ovarian reserve on milk production levels and concluded that they were not related.

One of the goals of biotechnology programmes is to obtain maximum performance in improving productive parameters, and one means to do so is to test for AMH-related genetic markers that would allow for the identification of high-genetic-merit males to genetically improve other parameters such as reproductive performance, health, and herd longevity in cows. The dosage of AMH or determination of AMH-related genetic markers could also be incorporated into government programme calculations, such us the USDA-Council on Dairy Cattle Breeding (CDCB) selecting sires with daughters with high AMH circulating levels (Jimenez-Krassel et al., 2015). These papers and others applied to buffalo contribute information that can be used to develop improvement programmes in the species based on reproductive biotechnologies, especially embryo transfer.

The apparently paradoxical results obtained here have also been reported by Jimenez-Krassel et al. (2009), who found no statistical differences in conception rates in cattle heifers with low AMH and AFC, despite the strong relationship with ovarian function (Mossa et al., 2012), endometrial development (Jimenez-Krassel et al., 2009), and oocyte quality (Bettegowda et al., 2007). These results are considered paradoxical as it would be thought that ovarian reserve, as the number of primordial follicles of a given female, must be correlated with reproductive performance, and this is the scenario that reproductive parameters to evaluate efficiency in reproduction in animal production systems should show. However, the reality is that reproductive success is a more complex phenomenon and reproductive efficiency should not be associated solely with one or two events or measurements. A successful cow or water buffalo has one calving a year, meaning that it needs only one successful oocyte from the given cohort of follicles consumed in each wave. The results reported here show that the evaluation of AMH levels is sufficient to do that. Indeed, the more important issue is not the number of potential oocytes, but the quality of the gamete. The hope of using AMH as a marker relies only upon the numerical aspects of the phenomena, and as a consequence more AMH means an extra number of primordial follicles. To date, the mathematical relationship of AMH levels with a given number of primordial or preantral follicles in the cow (number of oocytes for a given number of picograms or nanograms of circulating AMH) has not been reported. The differences observed in the calving period between Zebu cattle and water buffaloes, could also be associated with the need for activation of more follicles to conceive.

Many factors related to management, health, and environment could affect the emergence of a successful follicular wave with a competent oocyte for conception that is required for the beginning of a reproductive event. Age at first calving is influenced by many parameters, including weight, breed, and management regime (Grohn and Rajala-Schultz, 2000). It has been reported that in both species, the ovarian reserve is established at the age of 8 months, as the typical weaning period used in management in Columbia (Gutierrez et al., 2017). It can be concluded that the primordial follicles containing oocytes are ready to be successful earlier during the life of the animal than all other parameters needed for successful reproduction. More research is needed to establish the relationships that explain the quantitative aspects of the fact that competent oocytes are needed to meet the requirements of the parameters used in herd reproduction.

Table 1. Comparison of AMH levels and reproductive parameters between cattle and buffaloes. Average values of the parameters studied in the two populations of animals

		AMH (pg/mL)	Days open to pregnancy	Parity	Age at 1st calving (months)	Intercalving period
Bos indicus	**Mean**	1305.44	334	2.16	41.64	532.53
	SD	677.1	96.22	1.62	6.12	151.06
	Max	2226	603	6	59.7	1012
	Min	184	92	0	32.4	373
Bubalus bubalis	**Mean**	354.32	102.4	4.24	35.75	430.65
	SD	328.43	30.11	3.4	5.28	44.28
	Max	1856	188	12	48	569
	Min	78	47	1	26.6	346
	P value	<0.0001	<0.0001	<0.001	<0.0001	<0.001

The results of this study showed a lack of correlation between AMH levels and parity ($r=0.25$ $P=0.32$), while others have reported a negative correlation between age and AMH levels in humans ($r=0.30$, $P<0.05$) (Malisic et al., 2018). Recently, Vernunft et al. (2018), found the application of AMH in Holstein-Friesian heifers as a predictive and independent marker for fertility. They also analysed the effect of management factors on fertility and found no significant effects of the farm, age, weight, height, body condition score, progesterone levels and pregnancy status at blood sampling or their later milk yield, milk fat and protein on the AMH levels ($r<0.1$, $P>0.1$), and concluded that there were no correlations between AMH levels and age of first breeding or calving, services per conception as heifer or cow, days to first service, days open to pregnancy or conception rates.

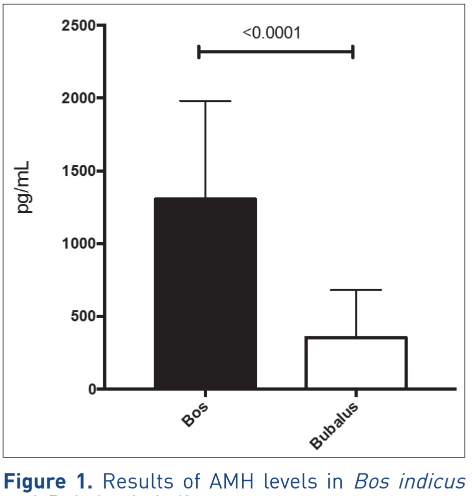

Figure 1. Results of AMH levels in *Bos indicus* and *Bubalus bubalis*

Souza et al. (2015) reported the use of AMH dosage in genetically superior dairy cattle to choose the best animals for superovulation treatments, and high numbers of the best quality and transferable embryos. The use of AMH in this manner has facilitated significant improvement given its association with oocyte quantity and quality and blastocyst yield (Stojsin-Carter et al., 2016), however, this remains a controversial issue. Batista et al. (2014) did not find correlations between AMH levels and follicular development in Nelore and Holstein cows, or in the parameters that involved related pregnancy and its outcome.

There is general agreement in their relationship with the number of follicles, but not with all other parameters used in reproductive biotechnology programmes. The identification of new biomarkers helps to improve the efficiency. In humans, Hamel et al. (2010) found a strong relationship between the expressions in granulosa cells of the genetic markers: UDP-glucose pyrophosphorylase-2 and pleckstrin homology-like domain, family A member 1 and pregnancy.

Guerreiro et al. (2018) used high-resolution mass spectrometry in follicular fluid samples from Nellore cows (*Bos indicus*) of two different conditions: high and low fertility, as determined by the number of oocytes produced, and found seven molecules candidates indicating producers of a high number of oocytes and two molecules indicating low producers. It is very interesting to note that though a given marker is associated with high production, its absence does not necessarily means low production. Therefore, researchers need to delineate markers of interest for each group, which may ultimately be related to biochemical pathways leading to higher or lower reproductive performance.

Certainly, the search for biomarkers is ongoing in different species, and it is crucial to address the differences in reproduction between animals and humans. Despite the lack of correlation between reproductive parameters and AMH, it is necessary to look for other biomarkers that could be used in animal selection, and in this case to apply them to buffaloes.

In reproduction, one event leads to the next, the sperm fertilizes the egg, and an embryo is formed, it will be transformed in a blastocyst that will implant producing pregnancy, and later a live born animal is delivered as a normal and natural process. However, is it possible to assign a unique characteristic of the process to the overall process? The reproductive process is highly complex, and low-quality oocytes are certainly not conducive to its beginning, while on the other hand, a good oocyte does not necessarily guarantee a live birth. Association can facilitate in this process, though it is important to remember that this is purely statistical.

Conclusions

AMH is viewed as representative of the follicular pool, and it is widely agreed that this pool is an indicator of the primordial follicular reserve, and serves as a better predictor of reproductive longevity than of the reproduction performance of the animals. It is possible to understand that biological parameters related specifically to ovulation follow the same pattern, and from human to farm animals, there are many aspects of the reproductive system that could confound the interpretation of results and affect the reproduction of the species. The results obtained here reinforce the fact that successful reproduction is a complex phenomenon and the reproductive process should be viewed differently, or using different tools that allow for an analysis of all the events needed to obtain the objective: live birth in a desired period of time.

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Razlike u razinama anti-Müllerovog hormona u krvnoj plazmi i reproduktivnih parametara između dvije vrste goveda *Bos indicus* i *Bubalus bubalis*

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Cilj je ovog rada bio procijeniti je li moguće ustanoviti povezanost između reproduktivnih parametara i razina anti-Müllerovog hormona (AMH) u dvije blisko srodne vrste goveda. Analizirane su razine AMH i reproduktivni parametri 50 bivola (*Bubalus bubalis*) i 50 zebu goveda (*Bos indicus*) iste dobi uzgojenih u sličnim uvjetima. Klinički podatci dobiveni su s farme u posebno osmišljenom pokusu. AMH je određivan pomoću ELISA metode. Rezultati pokazuju da su razine AMH više u goveda nego u bivola (P<0,0001). Bivoli su pokazali bolje reproduktivne parametre: servisno razdoblje (P<0,0001), paritet (P<0,0005), dob kod prvog teljenja (P<0,0001) i međutelidbeno razdoblje (P<0,0001). Premda obje vrste imaju različite reproduktivne parametre, nije ustanovljena povezanost između AMH i analiziranih reproduktivnih parametara niti u jedne od istraživanih vrsta. Zaključno, steone ženke goveda i bivola pokazuju tendenciju manjih razina AMH u usporedbi s nesteonim. Rezultati dobiveni u ovoj studiji su paradoksalni, jer vrste s nižim AMH razinama pokazuju bolje reproduktivne parametre. Ovi rezultati ukazuju na to da reproduktivni parametri unutar krda ne ovise samo o rezervi jajnih stanica u ženki.

Ključne riječi: *razina anti-Müllerov hormon, bivoli, zebu govedo, reproduktivni parametri*