Reproduction in Domestic Animals

Reprod Dom Anim doi: 10.1111/rda.12304
ISSN 0936–6768

Plasma Antimullerian Hormone as a Predictor of Ovarian Antral Follicular Population in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) Heifers

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Contents

In *Bos taurus* cattle, antimullerian hormone (AMH) has been demonstrated to have a high degree of correlation with ovarian antral follicle count and the number of healthy follicles and oocytes. To document the correlation between the plasma concentration of AMH and follicular number in *Bos indicus* and *Bos taurus* heifers, Nelore (*Bos indicus*, n = 16) and Holstein heifers (*Bos taurus*, n = 16) had their ovarian follicular waves synchronized. After synchronization, ovarian antral follicular population (AFP) was evaluated three times at 60-day (d) intervals (T-120 d, 120 days before plasma AMH determination; T-60 d, 60 days before; and T0, at the time of plasma AMH determination). The plasma AMH concentration was positively correlated with the number of ovarian follicles on the day of the follicular wave emergence in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers at each evaluation time ($p < 0.05$). The AFP was higher in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) heifers ($p < 0.05$). Similarly, the AMH concentration was higher in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) heifers ($p < 0.0001$). When heifers were classified as to present high or low AFP according to the mean of the AFP within each genetic group, high-AFP heifers presented a greater ($p < 0.0001$) AMH concentration than low-AFP heifers, regardless of the genetic group. In conclusion, the AFP is positively correlated with plasma AMH concentration in both *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. Furthermore, *Bos indicus* (Nelore) heifers presented both greater plasma AMH concentrations and AFP than *Bos taurus* (Holstein) heifers.

Introduction

Antimullerian hormone (AMH) is a member of the TGFβ superfamily of growth factors (Cate et al. 1986). Antimullerian hormone expression is high in granulosa cells of small antral follicles and decreases during terminal follicular growth (Vigier et al. 1984; Takahashi et al. 1986; Visser et al. 2007; Monniaux et al. 2008; Rico et al. 2009). Circulating AMH concentrations are positively associated with the total number of ovarian follicles in mice (Durlinger et al. 2002a) and in women (Fanchin et al. 2003). In *Bos taurus* cattle, AMH is also a reliable endocrine marker of the population of small antral gonadotropin-responsive follicles (Ireland et al. 2008; Rico et al. 2009).

Bos indicus cattle are the predominant breed raised in tropical regions. However, because *Bos indicus* cattle have subtle differences in their reproductive behaviour compared with *Bos taurus* breeds (Bó et al. 2003; Baruselli et al. 2007; Sartori et al. 2010), one cannot assume that the physiological parameters observed in *Bos taurus* would be the same as in *Bos indicus* cattle. Therefore, knowledge of the physiological differences between *Bos taurus* and *Bos indicus* breeds could be useful in developing reproductive strategies specific for each genetic group.

Among their many reported differences, *Bos indicus* females have a larger ovarian antral follicular population (AFP) per wave than *Bos taurus* (Segerson et al. 1984; Alvarez et al. 2000; Carvalho et al. 2008; Gimenes et al. 2008). The AFP in *Bos taurus* and *Bos indicus* cattle has an impact on ovarian function and management procedures associated with ovarian superstimulation and *in vivo* and *in vitro* embryo production (Pontes et al. 2010, 2011; Silva-Santos et al. 2011; Baruselli et al. 2012). Variations in circulating insulin and IGF-I concentrations have been associated with differences in AFP between *Bos indicus* and *Bos taurus* (Alvarez et al. 2000; Fortune et al. 2010; Sales 2011; Satrapa et al. 2013).

In *Bos taurus* cows, the measurement of AMH endocrine concentrations can help to predict both follicular and ovulatory responses to gonadotropin treatment. This information can be used as a predictor of the number of embryos a donor can produce (Monniaux et al. 2011). Yet, there is limited information about AMH concentrations and the variations in the total AFP between *Bos taurus* and *Bos indicus* cattle.

Thus, the present study was designed to compare the association between plasma AMH concentrations and AFP in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers maintained under the same management conditions. Furthermore, the present study evaluated whether a single plasma sample to measure AMH concentration taken at the time of follicular wave emergence can be a useful marker to predict the pool of ovarian follicles, regardless of genetic group. The hypotheses evaluated were (i) that AFP are positively associated with plasma AMH concentrations in both *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers and (ii) that both AFP and plasma AMH concentrations are greater in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) heifers.

Materials and Methods

Farm and heifers

The experiment was conducted at the Sao Paulo University Campus (USP, *campus* Pirassununga, SP, Brazil). A total of 32 cyclic (defined by the presence of *corpus luteum* during at least one of two consecutive

ultrasound examinations performed 14 days apart) heifers [16 *Bos indicus* (Nelore) and 16 *Bos taurus* (Holstein)]. The average weight was 354.5 ± 11.9 (T-120), 374.2 ± 10.5 (T-60) and 410.3 ± 11.2 (T0) to *Bos taurus* (Holstein) heifers; 420.1 ± 8.5 (T-120), 463.3 ± 6.7 (T-60), 503.2 ± 7.7 kg (T0), to *Bos indicus* (Nelore) heifers. The average weight gain was 465.1 g for day to *Bos taurus* (Holstein) heifers and 692.8 g for day to *Bos indicus* (Nelore) heifers._The body condition score [(1–5 scale, one indicating cachectic and five indicating obese (Ayres et al. 2009)] ranging between 3.0 and 3.5 at the first day of the trial were enrolled. All heifers were housed in dry lot pens and fed silage as forage and a corn and soybean meal-based concentrate. The ration was balanced to meet or exceed minimum nutritional requirements (NRC 2001). All procedures were approved by the Bioethics Commission of the School of Veterinary Medicine and Zootechny of the University of Sao Paulo, São Paulo (protocol number 2565/2012).

Experimental design

Heifers' AFP were evaluated at three different time periods, each 60 days (d) apart (T-120 d, 120 days before plasma AMH determination; T-60 d; 60 day before; and T0, at the time of plasma AMH determination). To synchronize the ovarian follicular wave emergence and to therefore evaluate the AFP, the heifers' oestrous cycles were synchronized using estradiol plus progesterone treatment. Four days before each ovarian follicular population evaluation, heifers received an intravaginal progesterone device (CIDR®; Zoetis, Sao Paulo, SP, Brazil) and intramuscular treatment with 2 mg of estradiol benzoate (EB; Sincrodiol®; Ourofino Agronegocio, Cravinhos, SP, Brazil). Based on previous scientific studies in both *Bos indicus* and *Bos taurus* cattle, the expected time frame for new follicular wave emergence was defined as 3–4 days after the estradiol plus progesterone treatment (Bó et al. 2002, 2003; Martínez et al. 2005; Caccia and Bó 2008; Carvalho et al. 2008; Sá Filho et al. 2011). To confirm the occurrence of the new follicular wave emergence, heifers had their ovaries examined daily to verify the development of a new dominant follicle [follicle ≥6.2 mm for *Bos indicus* (Nelore) (Gimenes et al. 2008) and follicle ≥8.5 mm for *Bos taurus* (Holstein) (Ginther et al. 2003)].

For purpose of analyses of the relationships between AFP and AMH concentration, heifers within each genetic group were classified into one of two AFP categories (Low or High) according to the average of the AFP in each genetic group [*Bos taurus* (23 folicles) or *Bos indicus* (38 follicles)].

Ultrasound examinations

Ovarian transrectal ultrasonographic examinations were performed using a 7.5 MHz linear-array transducer (Mindray DP - 2200 VET, Shenzhen, China) by the same technician. During each ultrasound evaluation, all antral follicles ≥3 mm in diameter on both ovaries were counted as part of the AFP.

Blood collection and hormonal assays

Blood samples were collected by jugular venipuncture on day T0. The samples were immediately placed on ice, and later centrifuged at 3000 × **g** for 15 min for plasma separation. Plasma samples were frozen at −25°C until later analysis. Plasma AMH was evaluated using an enzyme-linked immunosorbent assay (ELISA) kit (Ansh Labs, Webster, TX, USA). The sensitivity of the AMH assay was 0.011 ng/ml and intra-assay CV were <5%. All AMH assays were performed at the IgAc (Institute Genese of Scientific Analyses, Sao Paulo, Brazil).

Data analysis

All data were analysed with SAS *System for windows* and are presented as the means ± SEM, except for the correlation studies. Analyses of follicular populations were performed with PROC MIXED of SAS 9.3 (SAS Institute Inc., Cary, NC, USA). For the correlation studies, significance was ascertained by Bravais–Pearson r critical values performed PROC CORR of SAS 9.2 and PROC REG to obtain the regression functions. The data were analysed according to the number of follicles, follicular growth rate and day of follicular emergence in different times and plasma AMH (T0), considering in the statistical model only the effect of the genetic group. The repeatability of the ovarian follicular population results was calculated as the ratio of the between-animal variance to the sum of the between-animal and residual variances. For all analyses, differences with $p > 0.05$ were considered not significant. The analyses of the influence of the AFP categories (low and high) within each genetic group on the AMH concentration were performed with PROC GLIMMIX of SAS 9.3. For this analysis, the AMH concentration was transformed to SQRT. In the statistical model were included the fixed effects of genetic groups (*Bos indicus* or *Bos taurus*), AFP category (Low or High) and their interaction.

Results

The number of AFP was higher in the *Bos indicus* (Nelore) than in the *Bos taurus* (Holstein) heifers regardless of the time of evaluation (Fig. 1). The total number of follicles larger than 3 mm was highly variable

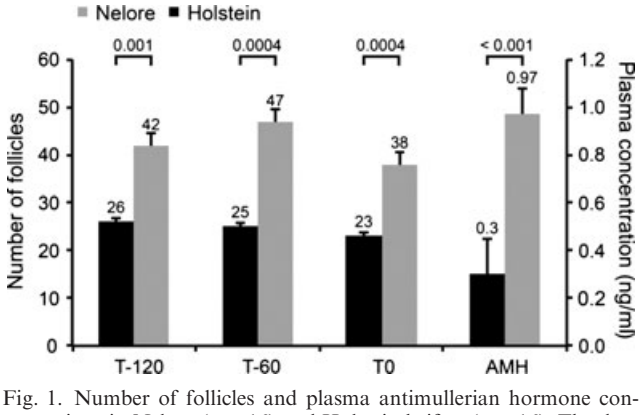

Fig. 1. Number of follicles and plasma antimullerian hormone concentrations in Nelore (n = 16) and Holstein heifers (n = 16). The data are the means ± SEM

between heifers. The AFP ranged from 18 to 85 in *Bos indicus* (Nelore) heifers and from 8 to 51 in *Bos taurus* (Holstein) heifers. However, the individual AFPs at 60-day intervals were highly repeatable. Within-animal repeatability (r) measured by this model was high for the AFP ($r = 0.85$). Furthermore, the plasma AMH concentration was not correlated with the follicular growth rate to *Bos indicus* (Nelore; $r = -0.16$, $p = 0.34$) and to *Bos taurus* heifers (Holstein; $r = 0.05$, $p = 0.75$). Similarly, the plasma AMH concentration was not correlated with the day of follicular emergence to Nelore ($r = 0.05$, $p = 0.75$) and to Holstein heifers ($r = 0.09$, $p = 0.57$).

The plasma antimullerian hormone concentration measured at T0 was highly correlated with the number of ovarian follicles detected by ultrasonography at T0, T-60 and T-120 in *Bos taurus* (Holstein) and *Bos indicus* (Nelore) heifers (Fig. 2). Regardless of the genetic group, heifers with high numbers of follicles had high plasma AMH concentrations in all periods evaluated (T0, T-60, T-120; Fig. 2). Yet, the overall AMH concentrations were higher in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) heifers (Fig 1). Furthermore, heifers classified with high AFP within each genetic group have greater AMH plasmatic concentration, regardless of the genetic group (Table 1).

Discussion

To our knowledge, this is the first comparative report of plasma AMH concentrations in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers and contributes new information regarding the differences in the folliculogenesis and reproductive physiology of these two genetic groups. The data have demonstrated that AMH could be a satisfactory endocrine marker of AFP in both *Bos indicus* (Nelore) and *Bos taurus* (Holstein) cattle. Moreover, plasma AMH concentration could be predictive of the ovarian status of heifers over a long-term period, as suggested in previous reports (Rico et al. 2009).

The number of ovarian antral follicles was higher in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) heifers at all evaluated periods. This difference is similar to the findings of previous scientific reports comparing the reproductive physiology of these different genetic

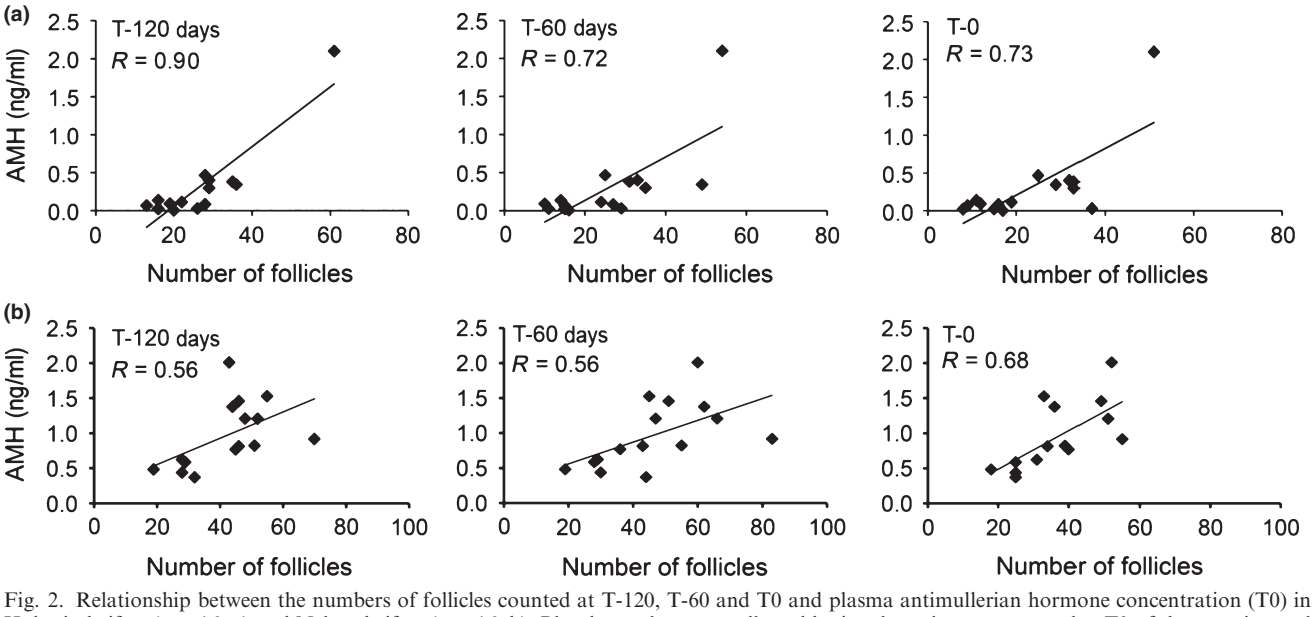

Fig. 2. Relationship between the numbers of follicles counted at T-120, T-60 and T0 and plasma antimullerian hormone concentration (T0) in Holstein heifers (n = 16; a) and Nelore heifers (n = 16; b). Blood samples were collected by jugular veinpuncture on day T0 of the experimental design

Table 1. Antral follicular population category and plasma AMH concentration in *Bos taurus* (Holstein) and *Bos indicus* (Nelore) heifers

	Genetic group				p-values		
	Bos taurus (Holstein)		*Bos indicus* (Nelore)				
AFP category[a]	Low AFP	High AFP	Low AFP	High AFP	Genetic group	AFP category	Genetic group*AFP category
No. heifers	8	7	8	7	–	–	–
No. follicles	13.4 ± 1.40	34.3 ± 3.12	28.4 ± 2.15	48.1 ± 2.33	<0.0001	<0.0001	0.80
Plasmatic AMH (ng/ml)[b]	0.06 ± 0.02	0.57 ± 0.26	0.78 ± 0.16	1.20 ± 0.16	<0.0001	0.001	0.26

[a]Antral follicle population (AFP) = numbers of follicle was counted to T0 of the experiment design. The AFP categories (Low and High) were established according the means of the number of follicle counted within each genetic group [*Bos taurus* (n = 23 folicles) or *Bos indicus* (n = 38 follicles)].
[b]Plasma Antimullerian hormone (AMH) was determined at T0 of the experimental design.

groups (Segerson et al. 1984; Alvarez et al. 2000; Carvalho et al. 2008). Additionally, high repeatability of the AFP of the same individual animals at 60-day intervals was observed, similar to previous studies (Boni et al. 1997; Burns 2005; Ireland et al. 2008).

A strong correlation between serum AMH levels and numbers of antral follicles is observed in women (Fanchin et al. 2003). In *Bos taurus* (Holstein), circulating AMH concentration is thought to be a reliable endocrine marker for predicting the relative number of morphologically healthy follicles and oocytes in ovaries (Rico et al. 2009). In the present study, regardless of genetic group, heifers presenting high AFP had high plasma AMH concentrations. Previous results showed that circulating AMH concentrations are higher for cattle with an intermediate or high antral follicle count compared to those with a low antral follicle count (Ireland et al. 2010). Furthermore, the number of ovarian follicles observed in all evaluation periods (T-120, T-60, and T0) was correlated with plasma AMH concentrations at T0 in both *Bos taurus* (Holstein) and *Bos indicus* (Nelore) heifers. These results suggest that AMH could be a possible long-term endocrine marker of ovarian activity similar to previously reported findings (Ireland et al. 2008; Rico et al. 2009). Therefore, a single blood sample taken at a random stage of the oestrous cycle to measure serum AMH concentration could be considered a reliable phenotypic marker to predict the relative number of follicles, regardless of genetic group.

As previously cited, AMH is produced by pre-antral and antral small follicles (Durlinger et al. 2002a). Despite the larger AFP observed in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) cattle, a previous report demonstrated that the primordial follicular populations were lower in *Bos indicus* heifers (Nelore) than in *Bos taurus* heifers (Angus) (Silva-Santos et al. 2011). Anti-mullerian hormone seems to be involved in mechanisms that inhibit the activation of primordial follicles to begin growing (Durlinger et al. 2002b; Fortune et al. 2010). Additionally, ovaries of AMH null mice contained significantly more early atretic follicles (Visser et al. 2007). It may be that the high plasma AMH concentration in *Bos indicus* heifers contributes to lower rates of follicular atresia resulting in the greater antral follicular populations. Yet, it is important to emphasize that the present study did not evaluate the pre-antral follicular population and further investigation are certainly required.

In conclusion, ovarian antral follicular populations are positively correlated with plasma AMH concentrations in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. Furthermore, *Bos indicus* (Nelore) heifers were found to have greater plasma AMH concentrations and larger ovarian antral follicular populations than *Bos taurus* (Holstein) heifers.

Acknowledgements

The authors would like to thank the staff at Pirassununga Campus – USP (Pirassununga-SP) for allowing the use of their animals and facilities during this study. The authors are also grateful to Genese© for the donation of AMH ELISA kits. This research was supported by FAPESP (2012/00889-5) and CAPES.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

EOS Batista: research design, analysis and interpretation of data, drafting the paper; GG Macedo: research design, drafting the paper; RV Sala, MDDV Ortolan, EF Jesus, RNVR Lopes, FP Rennó: acquisition of data; MF Sá Filho: interpretation of data, drafting paper and revising critically; TA Dell Vale: acquisition of data, analysed data; PS Baruselli: research design, interpretation of data, revising paper critically.

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Submitted: 21 Nov 2013; Accepted: 13 Feb 2014

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