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Comparison of Antral and Preantral Ovarian Follicle Populations Between *Bos indicus* and *Bos indicus-taurus* Cows with High or Low Antral Follicles Counts

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Contents
The objective was to compare populations of antral and preantral ovarian follicles in *Bos indicus* and *Bos indicus-taurus* cows with high and low antral follicle counts. Nelore (*Bos indicus,* n = 20) and Nelore X Angus (1/2 *Bos indicus-taurus,* n = 20) cows were subjected to follicular aspiration without regard to the stage of their oestrous cycle (day of aspiration = D0) to remove all follicles ≥3 mm and induce growth of a new follicular wave. Ovaries were examined by ultrasonography on D4, D19, D34, D49 and D64, and antral follicles ≥3 mm were counted. Thereafter, cows were assigned to one of two groups: high or low antral follicular count (AFC, ≥30 and ≤15 antral follicles, respectively). After D64, ovaries were collected after slaughter and processed for histological evaluation. There was high repeatability in the numbers of antral follicles for all groups (range 0.77–0.96). The mean (±SD) numbers of antral follicles were 35 ± 9 (*Bos indicus*) and 38 ± 6 (*Bos indicus-taurus)* for the high AFC group and 10 ± 3 (*Bos indicus*) and 12 ± 2 (*Bos indicus-taurus*) follicles for the low AFC. The mean number of preantral follicles in the ovaries of *Bos indicus-taurus* cows with high AFC (116 226 ± 83 156 follicles) was greater ($p < 0.05$) than that of *Bos indicus* cows (63 032 ± 58 705 follicles) with high AFC. However, there was no significant correlation between numbers of antral and preantral follicles.

Introduction

The number of bovine embryos produce by IVF is increasing in Brazil. This situation has been attributed to the large Brazilian cattle population (~200 million animals), composed largely of Nelore – *Bos indicus* – cows (80%). This breed has much higher rates of oocytes recoverable by ultrasound-guided follicular aspiration (OPU) than European breed – *Bos taurus* – (Stroud 2010). In that regard, the mean number of oocytes recovered per OPU procedure is 18–25 oocytes in *Bos indicus* breeds (Watanabe et al. 1999; Thibier 2004; Pontes et al. 2011) and 4–14 oocytes in *Bos taurus* breeds (Machado et al. 2003; Rubin et al. 2005; Martins et al. 2007).

There has been an increasing interest on crossbred cattle (*Bos indicus* × *Bos taurus*), because they may provide the best characteristics of the two types, namely disease and weather resistance (*indicus*) and meat and milk production (*taurus*; Pontes et al. 2010). However, there are apparently no reports regarding antral and preantral follicle population on *taurus-indicus* donors.

There is high variability in the population of ovarian preantral follicles (Erickson 1966a,b; Silva-Santos et al. 2011). Conversely, the number of antral follicles per follicular wave is repeatable within animals (Burns et al. 2005), and some cows present remarkably high versus low antral follicle populations during follicular waves (Ireland et al. 2008). However, there is a lack of information comparing preantral and antral follicular populations, particularly on *indicus* and *indicus-taurus* cross cows.

The objective of this study was to compare the number of preantral and antral follicles between *Bos indicus* and *Bos indicus-taurus* cows with high versus low antral follicle counts.

Materials and Methods

Cattle

Healthy, non-pregnant, non-lactating, cycling *Bos indicus* (Nelore, n = 20, 72–96 months old) and *Bos indicus-taurus* cross (1/2 Nelore X Angus, n = 20, 72–96 months old) cows were subjected to follicular aspiration without regard to the stage of the oestrous cycle (day of aspiration = D0). All follicles ≥3 mm were removed. Cows were maintained in *Brachiaria brizantha* pastures and given *ad libitum* access to mineral salt. The mean body condition score of cows was 3.5 ± 0.5 (scale of 1 to 5), and the average body weight was 450 ± 10 kg (Lowman et al. 1976).

Antral follicular counting

On D4, D19, D34, D49 and D64, ovaries were examined by ultrasonography (Áquila PRO, Pie medical, Maastricht, The Netherlands) using a 7.5-convex array transducer. Antral follicles ≥3 mm were counted as described (Burns et al. 2005; Ireland et al. 2008). After five evaluations, cows were assigned to one of two groups: high antral follicle count (AFC, mean ≥30 follicles, *Bos indicus,* n = 7; *Bos indicus-taurus,* n = 6) or low AFC (mean ≤15 follicles, *Bos indicus,* n = 6; *Bos indicus-taurus,* n = 6; Ireland et al. 2008). From 40 animals evaluated by ultrasonography, cows with intermediate AFC (16–31 follicles, n = 15) were eliminated from any further studies.

Collection of ovaries

After the last evaluation (D64), cows were slaughtered and ovaries were collected for histological evaluation. Following collection, ovaries were washed in 0.9% saline, cut longitudinally into halves and fixed in Bouin's fixative for 24 h. After being immersed in fixative, ovaries were transported to the laboratory. Ovaries were then placed in 70% alcohol. Only ovaries without corporea lutea (CL) were used to ensure good histological processing, and only one ovary per female was analysed. Ovarian halves were dehydrated in alcohol,

cleared with xylene, embedded in paraffin, and all the tissue was serially sectioned (7 μm) with a rotating microtome (Leica®, Wetzlar, Germany). In all ovaries, one from 120 histological sections (Cahill et al. 1979) was mounted and stained with periodic acid Schiff (PAS) and haematoxylin. All sections were used to evaluate the number of morphologically healthy follicles.

Preantral follicles were classified according to their stage of development as primordial (one layer of flattened or flattened–cuboidal granulosa cells surrounding the oocyte), primary (a layer of cuboidal granulosa cells around the oocyte) or secondary (oocytes surrounded by more than one complete layer of cuboidal granulosa cells) (Hulshof et al. 1994; Carámbula et al. 1999). Sections were examined using a light microscope (Nikon®, Tokyo, Japan), and the morphological appearance was evaluated, considering the integrity of the basal membrane, cell density, the presence or absence of pyknotic bodies and the integrity of the oocyte. On the basis of these parameters, only healthy follicles were further evaluated (Lucci et al. 2002).

Using an ocular micrometer, average diameters of oocytes were determined by measuring two follicles of each category (primordial, primary, and secondary) per section in which the nucleolus of the oocyte was observed (equatorial section). Each follicle and its associated oocyte were measured in two dimensions, and the arithmetic mean of the two measures was determined. The strategy for considering oocyte nuclei was important to avoid counting the same follicle in two sections. All procedures were performed by the same operator.

Estimate of the number of preantral follicles

The number of preantral follicles was estimated by counting all follicles in each histological section, as described (Silva-Santos et al. 2011); counting was performed by only one operator in a blinded trial. Only follicles in which the oocyte nuclei was visible in each histological section were counted. The nucleus of the oocyte was used as a marker, according to the correction factor described (Gougeon and Chainy 1987) and the following formula:

$$Nt = (No \times St \times ts)/(So \times do)$$

Nt = estimated total number of follicles of each category; No = number of follicles observed in the ovary; St = total number of cuts done in the ovary; ts = cutting thickness; So = total number of sections evaluated; and do = mean diameter of the follicle nucleus of each category.

Statistical analysis

Results are presented as mean ± SD. R software (2013) was used to test the normality of the samples. Because data were not normally distributed, a Mann–Whitney test was used to determine whether statistical differences existed among individual means. Correlation between antral and preantral follicle population was analysed with the Pearson's linear correlation test. Repeatability (proportion of the total variance that could be attributed to animal variance, range 0–1, 1 = perfect) was calculated (Boni et al. 1997). Significance was set at $p \leq 0.05$.

Results

After five ultrasound evaluations, there was repeatability in the number of antral follicles for all groups evaluated (*Bos indicus* with high AFC $r = 0.94$, and low AFC, $r = 0.96$; *Bos indicus-taurus* with high AFC $r = 0.87$, and low AFC, $r = 0.77$). The average number of antral follicles was 35 ± 9 for *Bos indicus* cows with high AFC and 10 ± 3 for the low AFC cows. For *Bos indicus-taurus* cows, the average was 38 ± 6 for those with high AFC and 12 ± 2 for the low AFC group (Table 1).

There was extreme variation among individuals within a group and between breeds. The number of follicles within *Bos indicus* cows with high AFC ranged from 10 719 to 152 999 preantral follicles, from 51 302 to 260 751 follicles for *Bos indicus-taurus* with high AFC, from 1754 to 62 348 follicles for *Bos indicus* cows with low AFC and 2909 to 320 504 follicles for *Bos indicus-taurus* with low AFC. The number of preantral follicles from *Bos indicus* cows with high AFC (63 032 ± 58 705) was less ($p < 0.05$) than that of *Bos indicus-taurus* cows with high AFC (116 226 ± 83 156; Table 2).

There was no significant correlation between the antral and preantral follicle populations. Some *Bos indicus* cows with high AFC (n = 5) had lower numbers of preantral follicles (10 719; 16 585; 27 690; 49 519; and 55 752 follicles) compared with the group average (63 032 follicles). Some crossbred cows with high AFC (n = 4) also had lower numbers of preantral follicles (57 596; 51 302; 70 503; and 85 954 follicles) than average (116 226 follicles). Furthermore, some cows with low AFC had greater numbers of preantral follicles than the average of the group both for some *Bos indicus* cows (n = 3; 30 975; 38 828; and 62 348 follicles; average of 24 265 follicles) and crossbred (n = 2; 99 696 and 320 504 follicles; average of 85 748 follicles).

Among the ovaries assessed (n = 25), 18 (72%) had multioocyte follicles (primordial, primary and second-

Table 1. Mean (±SD) number of antral follicles per ovary of *Bos indicus* and *Bos indicus-taurus* cows with high and low antral follicle count (AFC), assessed by ultrasonography

		No. of antral follicles per ovary					
Groups	n	US 1	US 2	US 3	Us 4	US 5	Mean
Bos indicus high AFC	7	32 ± 8	36 ± 9	37 ± 12	37 ± 12	34 ± 9	35 ± 9
Bos indicus low AFC	6	10 ± 3	9 ± 4	9 ± 4	11 ± 3	11 ± 4	10 ± 3
Bos indicus–taurus high AFC	6	35 ± 10	38 ± 8	37 ± 8	41 ± 11	38 ± 6	38 ± 6
Bos indicus-taurus low AFC	6	12 ± 4	12 ± 3	11 ± 2	13 ± 3	12 ± 2	12 ± 2

Ovaries ultrasound evaluation performed spaced 15 days apart.

Table 2. Mean (±SD) of preantral follicles from *Bos indicus* and *Bos indicus-taurus* cows of high and low antral follicle count (AFC)

Groups	n	Number of preantral follicles			
		Primordial	Primary	Secondary	Total
Bos indicus high AFC	7	43 623 ± 39 136[b]	21 017 ± 17 964[a]	4283 ± 4681[a]	63 032 ± 58 705[b]
Bos indicus low AFC	6	10 282 ± 14 393[c]	8219 ± 7912[ab]	9822 ± 11 199[a]	28 324 ± 24 525[b]
Bos indicus-taurus high AFC	7	109 370 ± 80 192[a]	6279 ± 3661[b]	577 ± 538[b]	116 226 ± 83 156[a]
Bos indicus-taurus low AFC	6	81 561 ± 128 402[ab]	3995 ± 3074[b]	149 ± 49[b]	85 748 ± 129 628[ab]

[a-c]Within a column, means without a common superscript differed ($p \leq 0.05$).

ary). Multioocyte follicles were observed in the ovaries of *Bos indicus* (n = 5) and *Bos indicus-taurus* cows (n = 6) with high AFC and also in those of *Bos indicus* (n = 4) and *Bos indicus-taurus* cows with low AFC (n = 3).

Discussion

After five successive ultrasound evaluations, *Bos indicus* and *Bos indicus-taurus* cows had repeatability (range 0.77–0.96) in the number of antral follicles, which allowed us to reliably assign these cows into groups with either high or low AFC. The repeatability in the number of antral follicles per follicular wave had already been reported in dairy cattle (Burns et al. 2005), but numbers of antral and preantral (ovarian reserve) follicles have apparently never been compared between *Bos indicus* and *Bos indicus-taurus*.

The average number of antral follicles in *Bos indicus* (10 ± 3) and *Bos indicus-taurus* (12 ± 2) cows with low AFC was similar to the average number reported in a previous study for *Bos taurus* cows with low AFC (10.9 ± 1.1; Ireland et al. 2009). However, the average number of antral follicles in *Bos indicus* (35 ± 9) and *Bos indicus-taurus* (38 ± 6) animals with high AFC was greater than numbers reported for *Bos taurus* animals with high AFC (27.3 ± 1.2; Ireland et al. 2009).

Bos indicus cattle have more follicular waves (Viana et al. 2000), greater numbers of follicles per follicular wave (Carvalho et al. 2008) and more oocytes recovered by follicular aspiration (3 to 4 times greater; Rubin et al. 2005; Martins et al. 2007) than *Bos taurus* cattle. In addition to these differences between *Bos indicus* and *Bos taurus* breeds, cattle with low AFC during follicular waves have greater blood FSH concentrations. In addition, high variation in the number of antral follicles during follicular waves was associated with expression of genes involved in estradiol production by granulosa cells (CYP19A1), adjustment of FSH action (anti-Müllerian hormone – AMH), differentiation and functioning of thecal cells (TBC1D1), estradiol responsiveness (ESR1, ESR2) and determinants of oocyte quality in cumulus cells (CTSB; Ireland et al. 2009).

There was high variation in the number of preantral follicles in *Bos indicus* and *Bos indicus-taurus* cows; this variation was similar to that described in previous reports (Tanaka et al. 2001; Silva-Santos et al. 2011), with ranges of 0 from 700 000 preantral follicles (Erickson 1966a). In a recent study, the number of preantral follicles was not significantly different between *Bos indicus* and *Bos taurus* females at various ages (Silva-Santos et al. 2011). However, in the present study, preantral follicle populations were significantly different between *Bos indicus* and *Bos indicus-taurus* cows with high AFC and between *Bos indicus* cows with low AFC and *Bos indicus-taurus* cows with high AFC. The average number of preantral follicles of *Bos indicus-taurus* cows with high AFC (116 226 follicles) was greater than that of *Bos indicus* cows with high AFC (63 032 follicles; Table 2). The average percentage of primary and secondary follicles in *Bos indicus* cows was greater (40% for high and 64% for low AFC group) compared with *Bos indicus-taurus* cows (5.5% for high and 5% for low AFC group), suggesting different mechanisms during the follicular transition.

Although there are no apparent studies comparing follicular populations between breeds, the average number of preantral follicles reported for Angus heifers (109 613 follicles; Silva-Santos et al. 2011) was slightly lower than those reported for Hereford heifers (132 000 follicles; Erickson 1966a). However, for cows, the average of Angus cows (89 577 follicles; Silva-Santos et al. 2011) was greater than that of Hereford cows (22 000 follicles; Erickson 1966a). Thus, we suggest that the influence of the Nelore and *Bos taurus* breeds may have contributed to the size of the follicular ovarian reserve.

In the present study, there was no significant correlation between the number of antral and preantral follicles. More than half of the cows with high AFC (*Bos indicus*, n = 5; *Bos indicus-taurus*, n = 4) had a lower number of preantral follicles compared with the average of the group. Similarly, some *Bos indicus-taurus* cows with low AFC (n = 2) had lower numbers of preantral follicles compared with the average. However, one female of the group *Bos indicus-taurus* with low AFC had a greater number of preantral follicles (320 504 follicles) than did the cows of the high AFC group for both *Bos indicus* (63 032 follicles) and *Bos indicus-taurus* cows (116 226 follicles). So far, we cannot explain the absence of correlation between the number of antral and preantral follicles. We believe that the complexity of this aspect may be due to the variation in standard deviation, as well as the large variation among animals.

In conclusion, numbers of antral follicles in the ovaries of *Bos indicus* and *Bos indicus-taurus* cows with high and low AFC were repeatable during follicular waves. There were differences in ovarian reserves between the cows; *Bos indicus-taurus* cows with high AFC had greater number of preantral follicles than did *Bos indicus* cows with high AFC. However, there is no

significant correlation between antral and preantral populations. We inferred that the influence of the Nelore breed of cattle and of the *Bos taurus* breed contributed to these differences.

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Conflict of interest

Authors declare no conflict of interest.

Author contributions

Silva-Santos, KC - writing and editing, ovarian processing, slides evaluation Santos; GMG - OPU, ovaries collection and ultrassonography Siloto; LS - ovarian collection and processing Marcantonio; TN - OPU, ovaries collection and ultrassonography Morotti; F - OPU, ovaries collection and ultrassonography Seneda, MM - supervisor.

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