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The correlation between the number of antral follicles and ovarian reserves (preantral follicles) in purebred *Bos indicus* and *Bos taurus* cows

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ABSTRACT

The objectives of this study were to compare populations of preantral follicles between purebred *Bos indicus* and *Bos taurus* cows with high or low antral follicle counts (AFC) and to correlate the number of preantral follicles with the population of antral follicles. Nelore (*Bos indicus*, $n = 100$) and Angus (*Bos taurus*, $n = 100$) cow ovaries were collected at abattoirs and examined using ultrasonography. Antral follicles ≥3 mm were counted, and the cows ovaries were assigned to high (G-High) or low (G-Low) AFC groups based on the mean number (±1 SD) of ovarian antral follicles: *Bos indicus* with high AFC (≥57 follicles, $n = 8$) or low AFC (≤21 follicles, $n = 8$) and *Bos taurus* with high (≥45 follicles, $n = 10$) or low AFC (≤13 follicles, $n = 10$). The ovaries were processed, and the number of preantral follicles was estimated. Between-groups comparisons were performed using a Kruskal-Wallis test, and the correlation between preantral and antral follicles was evaluated using a Pearson's correlation test ($P \leq 0.05$). A large variation in the number of preantral follicles was observed among the animals. Although there was a correlation between the population of preantral follicles and the number of antral follicles, there was no difference between the mean number of preantral follicles in the *Bos indicus* G-High (48,349 ± 30,149) and G-Low groups (33,037 ± 31,710) or between the *Bos taurus* G-High (35,050 ± 36,060) and G-Low groups (30,481 ± 43,360). Therefore, the preantral follicle population did not differ between purebred *Bos indicus* and *Bos taurus* cattle with high or low AFC but was correlated with the number of antral follicles. In addition to the large within-groups variation in the number of preantral follicles, some cows with high AFC had lower populations of preantral follicles compared to the low AFC group, and the highest population of preantral follicles was observed in both *Bos indicus* and *Bos taurus* with low AFC.

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1. Introduction

More oocytes can be obtained by *ovum pick up* (OPU) in *Bos indicus* compared to *Bos taurus* cattle (Pontes et al., 2009, 2010), and researchers wish to identify the underlying mechanism that determines this larger oocyte production. *Bos indicus* cows typically have many follicular waves (Figueiredo et al., 1997; Viana et al., 2000), follicles per wave (Carvalho et al., 2008) and antral follicles <5 mm in diameter (Segerson et al., 1984) but small corpora lutea (CL; Rhodes et al., 1995; Sartorelli et al., 2005) compared to *Bos taurus* females. However, no studies have explained the differences in oocyte production between these breeds.

Variation in the number of antral follicles in female bovine ovaries has been associated with anti-Müllerian hormone (AMH) concentrations (Ireland et al., 2008, 2009; Rico et al., 2009,2011; Ereno et al., 2012). AMH has been used to predict ovarian response to a stimulatory treatment in women (Van Rooij et al., 2002; Gruijters et al., 2003; Broer et al., 2009) and antral follicle count (AFC) has also been used to predict poor ovarian response prior to *in vitro* fertilization (IVF) in women with the identical level of accuracy and clinical value as AMH (Hendriks et al., 2005, 2007; Broer et al., 2009). In cattle, both AMH and AFC can be used to determine embryo production capacity (Monniaux et al., 2010; Rico et al., 2009, 2011, 2012). The number of antral follicles observed during a follicular wave is a repeatable observation (Singh et al., 2004; Burns et al., 2005; Ireland et al., 2007, 2008, 2009, 2011; Silva-Santos et al., 2014a,b), which allows females to be ranked according to reproductive capacity using transvaginal ultrasound. An earlier study has also shown that cows with a low AFC exhibit lower fertility (Mossa et al., 2012).

Preantral follicle numbers are also highly variable among females (Erickson, 1966; Silva-Santos et al., 2011,2014a,b). A correlation has been observed between the antral follicle population and the number of healthy follicles and oocytes in *Bos taurus* females with high and low AFC (Ireland et al., 2008). However, a comparison between different purebred cattle with high vs. low AFC was not conducted in this study. We hypothesize that there is a correlation between populations of preantral and antral follicles in cattle with high or low AFC. Therefore, the aim of the present study was to compare ovarian populations of preantral follicles in purebred *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) females with high or low AFC and to correlate the preantral follicle population with the number of antral follicles.

2. Materials and methods

2.1. Ovary collection and antral follicular count

Ovaries ($n = 200$) from 72- to 96-month-old cycling and non-pregnant purebred cows (Nelore, *Bos indicus,* $n = 100$, and Aberdeen Angus, *Bos taurus*, $n = 100$) were collected at abattoirs. Prior to slaughter, the females were maintained on cultivated pasture and fed mineral salt *ad libitum*. At slaughter, the mean body condition was 4 ± 0.5 (scale, 1–5; Lowman et al., 1976). All cattle were carefully evaluated according to body condition and health parameters before slaughter. The ovaries were transported to the laboratory in saline at 32–35 °C within three hours of collection. Immediately upon arrival, the ovaries were evaluated using ultrasonography, and follicles ≥3 mm were counted (Silva-Santos et al., 2014a,b). Each ovary was scanned with a 7.5-mHz convex-array transducer (Áquila PRO, Pie Medical, Maastricht, The Netherlands) from end-to-end to count antral follicles ≥3 mm. The AFC per pair of ovaries was determined for each animal. Following the ultrasound evaluation, the females were assigned to two groups according to the number of antral follicles ≥3 mm: females with a high (G-High) or low AFC (G-Low). The number of follicles per group (G-High vs. G-Low) was defined using the mean number of antral follicles per animal ± 1 standard deviation (SD): Nelore cows with G-High (≥ 57 follicles; $n = 8$) or G-Low (≤ 21 follicles; $n = 8$) and Aberdeen Angus cows with G-High (≥ 45 follicles; $n = 10$) or G-Low (≤ 13 follicles; $n = 10$). Animals with an intermediate AFC (Nelore, 22–56 follicles; Angus, 14–44 follicles) were eliminated from further analysis.

Following the antral follicle count, the ovaries were halved longitudinally and fixed in Bouin's fixative for 24 h. The ovaries were then placed in 70% alcohol. For an estimate of the preantral follicles, only ovaries without CL were used to provide histological sections with ovarian parenchyma and follicular population. Only one ovary per female was analyzed (Silva-Santos et al., 2011).

2.2. Histological evaluation and preantral follicle classification

The ovarian halves were dehydrated in alcohol, cleared with xylene and embedded in paraffin, and all tissue was serially sectioned at 7 μm with a rotating microtome (Leica®, Wetzlar, Germany). In all ovaries, each 120º histological section (Cahill et al., 1979) was mounted and stained with periodic acid Schiff (PAS) and hematoxylin. All sections were used to evaluate the number of healthy follicles. Preantral follicles were classified according to developmental stage, primordial (one layer of flattened or flattened-cuboidal granulosa cells surrounding the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by more than one complete layer of cuboidal granulosa cells; Hulshof et al., 1994; Carámbula et al., 1999) and as normal or degenerated according to their morphological appearance. The follicles were considered degenerated if they had one or more of the following aspects: a condensed oocyte nucleus, a shrunken oocyte, pyknotic bodies in the granulosa cells, low cellular density, or basement membrane breakdown. Based on these parameters, only morphologically healthy follicles were evaluated (Lucci et al., 2002). The sections were examined and photographed using a light microscope (Nikon®, Tokyo, Japan). Using an ocular micrometer, we determined the mean diameters of the oocytes by measuring two follicles in 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 used to identify oocyte nuclei was important to

Table 1
Mean (± SD) number of preantral follicles per ovary and antral follicles per female of *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) cows (72–96 mo) with high (G-High) vs. low (G-Low) AFC.

Groups	No. preantral follicles per ovary				No. antral follicles per female
	Primordial	Primary	Secondary	Total	
Bos indicus G-High AFC	31,639[a]	12,714	3,996	48,349	663 ± 8[a]
(≥ 57 follicles, *n* = 8)	(± 22,398)	(± 14,341)	(± 3,288)	(± 30,149)	
Bos indicus G-Low AFC	18,766[ab]	9,116	5,155	33,037	115 ± 5[b]
(≤ 21 follicles, *n* = 8)	(± 18,493)	(± 9,359)	(± 6,794)	(± 31,710)	
Bos taurus G-High AFC	19,777[ab]	11,337	4,174	35,288	552 ± 7[a]
(≥ 45 follicles, *n* = 9)	(±25,412)	(±11,568)	(±2,893)	(± 38,239)	
Bos taurus G-Low AFC	11,273[b]	15,617	3,591	30,481	111 ± 3[b]
(≤ 13 follicles, *n* = 10)	(±20,713)	(± 21,383)	(± 3,200)	(± 43,360)	

[a–c] Within a column, means without a common superscript differ ($P \leq 0.05$).

prevent the identical follicle from being counted in two sections. All procedures were performed by one operator.

2.3. Preantral follicle estimation

The number of preantral follicles was estimated by counting all follicles in all histological sections that corresponded to the entire ovary. Counting was performed by one operator in a blind trial. To avoid counting one follicle twice within a section, the border of the histological section was marked with a pen. The evaluation began from this point and followed a clockwise direction until the cortical portion was evaluated entirely. A follicle was counted only if the oocyte nucleus was visible in that histological section. The nucleus of the oocyte was used as a marker, according to the correction factor described by Gougeon and Chainy (1987) and the following formula: Nt = (No × St × ts)/(So × do), where Nt = the estimated total number of follicles in each category; No = the number of follicles observed in the ovary; St = the total number of cuts performed on the ovary; ts = the cutting thickness; So = the total number of sections evaluated; and do = the mean diameter of the follicle nucleus for each category.

2.4. Statistical analysis

The results are presented as the means ± SD. Minitab 16 was used to test sample normality. The numbers of antral follicles were compared using a Kruskal-Wallis test with the Dunn test for between-group comparisons. The correlation between antral and preantral follicles was analyzed using a Pearson's Linear Correlation test and a log-10 transformation. For all analyses, at least a 5% level of probability was required.

3. Results

Based on the ultrasound evaluation of 200 ovaries from *Bos indicus* (*n* = 100) and *Bos taurus* females (*n* = 100), the mean number of antral follicles was 63 ± 8 (G-High AFC) and 15 ± 5 (G-Low AFC) in *Bos indicus* cows and 59 ± 23 (G-High AFC) and 11 ± 3 (G-Low AFC) in *Bos taurus* females (Table 1).

The mean number of preantral follicles per ovary was similar ($P > 0.05$) for *Bos indicus* and *Bos taurus* females. There were 48,349 ± 30,149 (mean ± SD) preantral follicles in *Bos indicus* ovaries with high AFC and 33,037 ± 31,710 in those with low AFC. In *Bos taurus* females, there were 35,050 ± 36,060 and 30,481 ± 43,360 preantral follicles in ovaries with high and low AFC, respectively (Table 1).

The number of preantral follicles varied among individuals within a group (high or low) and breed (Fig. 1). Variation within Nelore females ranged from 13,660 to 87,638 (G-High) and 2462 to 93,560 follicles (G-Low). For Angus females, the number of preantral follicles ranged from 5388 to 120,493 (G-High) and 1348 to 147,006 (G-Low; Fig. 1). We observed contrasting situations for preantral and antral follicle populations, as shown in Fig. 1. Some animals with low AFC presented higher numbers of preantral follicles and vice versa. Additionally, in 37.5% of G-High AFC Nelore cows, the mean number of preantral follicles was smaller than that of the mean G-Low population, and 25% of G-Low AFC Nelore cows had the largest mean number of preantral follicles compared to the mean population of the G-High Nelore group. For Angus cows, 60% of the G-High AFC group had the smallest population of preantral follicles compared to the mean number of G-Low in Angus, and 20% of the G-Low AFC preantral follicle population in Angus cows was greater than the mean number in the G-High Angus group (Fig. 1).

Despite the within-group individual variation for the number of follicles, AFC was positively correlated with the number of primordial follicles ($r = 0.43$; $P < 0.05$) and the total number of preantral follicles in the ovaries ($r = 0.38$; $P < 0.05$; Table 2).

Among the ovaries assessed (*n* = 26), 14 (54%) had multi-oocyte follicles (primordial, primary and secondary). Multi-oocyte follicles were observed in 25% of *Bos indicus* with high AFC, 50% of *Bos indicus* with low AFC, 40% of *Bos taurus* with high AFC and 40% of *Bos taurus* with low AFC. The number of oocyte nuclei varied (2–5) within the multi-oocyte follicles. Ovigerous cords were observed in two *Bos taurus* cows: one from the high AFC group and one from the low AFC group (Fig. 2).

The histological classifications of polyovular follicles and ovigerous cords. Ovigerous cords (A) and multi-oocyte follicles (B) in the ovary of a *Bos taurus* cow with low AFC and multi-oocyte follicles in the ovary of a *Bos indicus* cow with low AFC (C). The presumptive nucleus of oogonias (arrows) within the ovigerous-like cords and presumptive nucleus of oocytes (Nu) were enclosed within a follicle-like cell with a single layer of granulosa cells (GC). The

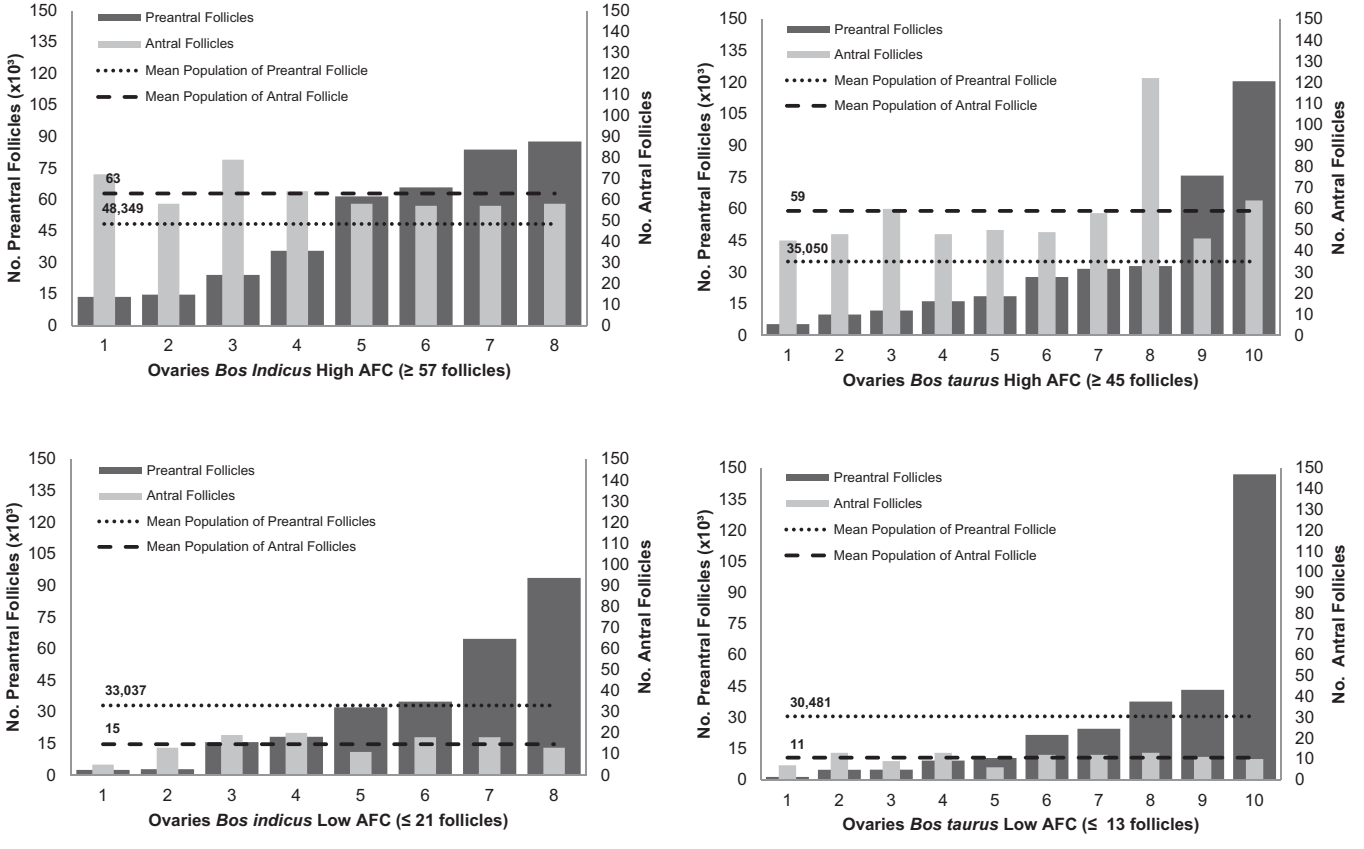

Fig. 1. Variation in the preantral follicle population among individuals of the same group (G-High vs. G-Low AFC) and between breeds (Nelore–*Bos indicus* vs. Aberdeen Angus–*Bos taurus*).

Table 2
Pearson correlation coefficients for populations of preantral and antral follicles.

	AFC	No. primordial follicles	No. primary follicles	No. secondary follicles	No. total preantral follicles
AFC	1	0.43*	0.18	0.24	0.38*
No. Primordial follicles		1	0.67***	0.40*	0.90***
No. Primary follicles			1	0.75***	0.88***
No. Secondary follicles				1	0.70***
No. Total preantral follicles					1

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

sections were stained with periodic acid Schiff (PAS) and hematoxylin. Bars = 50 μm. Original magnification X400.

4. Discussion

To our knowledge, this is the first comparative study examining populations of preantral ovarian follicles subsequent to antral follicle counts in ovaries retrieved from many cycling and non-pregnant purebred cows (Nelore–*Bos indicus* and Aberdeen Angus–*Bos taurus*) at slaughter. Although there are remarkable variations in the number of antral follicles during follicular waves among cattle, repeatability in antral follicle populations allows the identification of cattle with high or low antral follicle counts based on AFC (Singh et al., 2004; Burns et al., 2005; Ireland et al., 2007, 2008, 2011; Silva-Santos et al., 2014a,b). The antral follicle population is constant within individuals regardless of age, season, lactation stage or management conditions (Burns et al., 2005; Ireland et al., 2007, 2008; Silva-Santos et al., 2014a,b). AFC has an identical accuracy level and clinical value as AMH in predicting responses to assisted reproduction therapy (ART) in women (Hendriks et al., 2005; Broer et al., 2009) and can be used to predict the number of antral follicles in *Bos indicus* and *Bos taurus* females (Batista et al., 2014). These results suggest that AFC can be used in cattle because it is an inexpensive, easy-to-use tool that can improve field results.

In the present study, the mean number of preantral follicles was similar for *Bos indicus* and *Bos taurus* females with high and low AFC, although the ovaries were obtained from many cows (100 per breed) and the high or low AFC groups that only included females with antral follicle populations corresponding to extremes of 8% in *Bos indicus* and 10% in *Bos taurus*. Similar to previous studies by our group (Silva-Santos et al., 2011, 2014a,b), the preantral follicle populations showed considerable between- (Nelore and Aberdeen Angus of different ages – fetuses, heifers and cows; and Nelore and 1/2 Nelore × Angus cows with high or low AFC) and within-group variation among females. Conversely, differences have been observed in the number of preantral follicles in *Bos taurus* females between high and low AFC groups (Ireland et al., 2008). Breed might have generated this difference. In the present study, we evaluated the ovaries of purebred cattle that were genetically selected over several generations, whereas an earlier study compared preantral follicle populations between crossbred beef cattle. *Bos taurus* females within the high AFC group presented herein had ≥45 antral follicles and corresponded to 10% of Angus cows, whereas Ireland et al. (2008) reported ≥25 antral follicles, which corresponded to approximately 20% of the total individuals.

Previous studies have reported the total number of preantral follicles in cattle ovaries (Erickson, 1966; Ireland et al., 2008; Silva-Santos et al., 2011), but none have

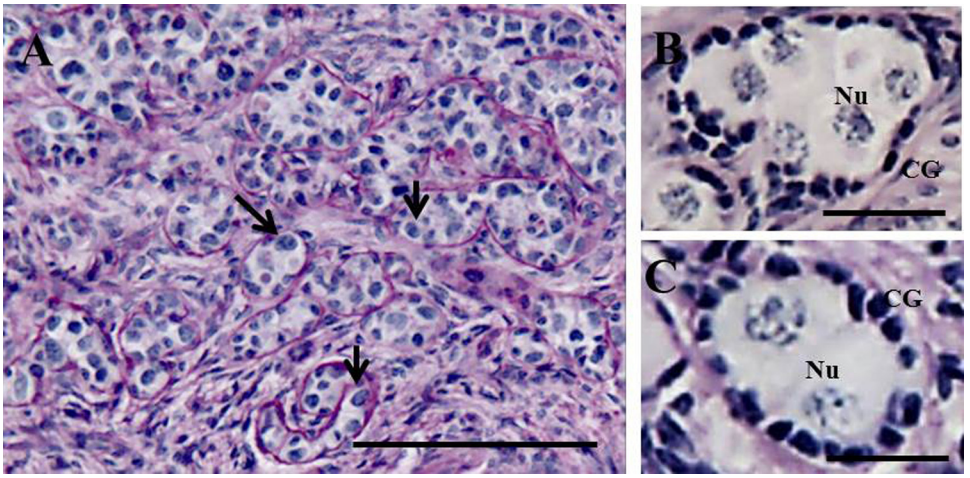

Fig. 2.

compared the ovarian reserve (preantral follicle population) of two purebred beef cows with remarkably high or low AFC selected from many animals. The mean number of preantral follicles in *Bos taurus* females with high AFC (35,050 follicles) in the present study was slightly higher than previously reported for *Bos taurus* cows with high AFC (29,056 follicles; Ireland et al., 2008).

In this study, we confirmed that preantral follicle populations were similar between *Bos indicus* and *Bos taurus*, regardless of the number of antral follicles in the ovaries, breed or age (Silva-Santos et al., 2011). However, there was a correlation between the AFC and number of primordial and total preantral follicles (Table 2). G-High AFC cows had an average of 15,312 (*Bos indicus*), or ~32%, and 4,807 (*Bos taurus*), or ~14%, more follicles than G-Low AFC cows. Most likely, the large variation in the number of preantral follicles accounted for this situation, which makes statistical comparison difficult, as has been previously observed in most bovine ovaries (Silva-Santos et al., 2011). The correlation observed between AFC and the primordial and total preantral follicles was to be expected because primordial follicles account for the majority of the preantral follicle population. However, although the cows were methodically assigned to groups of high or low AFC, the proportion of primordial follicles to total preantral follicles in *Bos indicus* (65% in the High- and 57% in the Low-AFC group) was similar to the result observed previously in *Bos indicus* cows, regardless of AFC (62%; Silva-Santos et al., 2011). In this context, it is noteworthy that *Bos indicus females* have more follicular waves (Viana et al., 2000), more follicles per wave (Carvalho et al., 2008) and more oocytes recovered by OPU (Pontes et al., 2009, 2010) than *Bos taurus* females. Furthermore, fewer antral follicles are associated with lower reproductive performance and suggest suboptimal fertility (Burns et al., 2005; Ireland et al., 2007, 2008, 2009, 2011; Jimenez-Krassel et al., 2009; Mossa et al., 2012). However, whether a high number of antral follicles per follicular wave is positively associated with fertility is still to be determined.

It has been previously suggested the oocyte generation in postnatal mammalian adult ovaries (Johnson et al., 2004, 2005; Zou et al., 2009) and the epigenetic regulation of histone 3 at lysine 4 (H3K4) in folliculogenesis (Seneda et al., 2008), as occurs in mammalian spermatogenesis (Godmann et al., 2007). Additionally, the lineages of *Bos indicus* and *Bos taurus* females may have influenced our results. Large variations were observed within both groups (high and low) although the individuals used in this study were purebred, and only 8–10% of cows at each extreme (high or low AFC) were selected. Some cows with high AFC (37.5% in Nelore and 60% in Angus) had the lowest preantral follicle populations compared to the mean of the low AFC group, and some females with low AFC (25% in Nelore and 20% in Angus) had more preantral follicles compared to the mean of the high AFC group (Fig. 1). The first scenario (females with high antral follicle counts and few preantral follicles) is noteworthy. Why the numbers of antral follicles are repeatable in cows with low numbers of preantral follicles is not clear. The opposite observation (females with low antral follicle counts and many preantral follicles) can be understood. In this scenario, females do not recruit these follicles to grow and develop until the antral stage. In addition to these observations, the highest population of preantral follicles was observed in both *Bos indicus* and *Bos taurus* cows in the low AFC group.

In the present study, we observed ovigerous cords and multi-oocyte follicles in *Bos indicus* and *Bos taurus* cows with high and low AFC (Fig. 2). The highest frequency of multi-oocyte follicles was observed in *Bos indicus* ovaries with low AFC (50%). Conversely, in an earlier study, higher frequencies of multi-oocyte follicles were observed in *Bos taurus* fetuses, heifers and cows compared to *Bos indicus* cattle of identical age (Silva-Santos et al., 2011). These structures are typically described in fetuses undergoing primordial follicle formation (Diniz et al., 2005; Yang and Fortune, 2008; Silva-Santos et al., 2011). Currently, the roles of ovigerous cords and multi-oocyte follicles in the ovaries of adult cows are not well understood, although they have both been reported in young and adult cattle (Ireland et al., 2008; Silva-Santos et al., 2011)

In conclusion, although there was a correlation between preantral and antral follicle populations, there was no difference in the number of preantral follicles between *Bos indicus* and *Bos taurus* cows with high and low numbers of antral follicles during follicular waves, which may be because of the large variation in the number of preantral follicles among individuals in the identical group. Furthermore, some cows with high AFC presented few preantral follicles compared to the low AFC group, while the highest population of preantral follicles was observed in cows with low AFC.

Conflict of interest

The authors declare no conflict of interest.

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