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Estimate of the population of preantral follicles in the ovaries of *Bos taurus indicus* and *Bos taurus taurus* cattle

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Abstract

The number of oocytes recovered from *Bos taurus indicus* females subjected to ovum pick-up averaged two to four times greater compared to *Bos taurus taurus* females. The objective of the present study was to test the hypothesis that this difference in oocyte yield was due to more preantral follicles in the ovaries of *Bos indicus* females. Ovaries (n = 64) from Nelore (*Bos indicus*) fetuses (n = 10), heifers (n = 12), and cows (n = 10), and Aberdeen Angus (*Bos taurus*) fetuses (n = 10), heifers (n = 12), and cows (n = 10) were cut longitudinally into halves, fixed, and processed for histological evaluation. The number of preantral follicles was estimated by counting them in each histological section, using the oocyte nucleus as a marker and employing a correction factor. The average number of preantral follicles in the ovaries of *Bos indicus* vs *Bos taurus* was (mean ± SD) 143,929 ± 64,028 vs 285,155 ± 325,195 for fetuses, 76,851 ± 78,605 vs 109,673 ± 86,078 for heifers, and 39,438 ± 31,017 vs 89,577 ± 86,315 for cows ($P > 0.05$). The number of preantral follicles varied greatly among individual animals within the same category, as well as between breeds. In conclusion, we inferred that the higher oocyte yield from *Bos indicus* females was not due to a greater ovarian reserve of preantral follicles. Therefore, mechanisms controlling follicle development after the preantral stage likely accounted for differences between *Bos indicus* and *Bos taurus* females in number of oocytes retrieved at ovum pick-up.

Keywords: Preantral follicle; Fetus; Bos indicus; Bos taurus; Bovine

1. Introduction

In recent years, Brazil has become the leading country in the world for the number of embryos produced *in vitro* [1]; this was attributed to the high number of follicles and oocytes recovered from *Bos taurus indicus* females [2]. This aspect has stimulated researchers to investigate reasons for differences between *Bos indicus* and *Bos taurus* females. It was reported that *B. indicus* females have more follicular waves [3,4], more follicles per wave [5], and a greater population of antral follicles < 5 mm in diameter than *Bos taurus taurus* females [6]. Furthermore, dominant follicles and corporea lutea (CL) are smaller and estrus is shorter in *B. indicus* compared with *B. taurus* females [7,8].

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However, none of these comparisons is as relevant as the production of oocytes that can be obtained from *Bos indicus* (Zebu) females. Intriguingly, hundreds of oocytes are routinely obtained in each ovum pick-up (OPU) from *Bos indicus* females. For instance, our team harvested 251 oocytes from a Nelore cow in a single OPU session (Seneda et al, unpublished results), and there are reports of up to 564 oocytes from a female of this same breed. Beside the number of oocytes (564), the quality of these oocytes collected is also impressive, since the majority (77%) were classified as viable (Grades I, II, and III) [9]. The average number of oocytes harvested from Nelore cows per OPU session, which ranged from 18 to 25 recovered oocytes, was frequently reported to be three to four times higher than the average described for *Bos taurus* females [10–12]. We recently reported repeated harvests of 60 oocytes from Zebu donors using subsequent OPU sessions, without hormone stimulation or synchronization of follicular growth [2].

Despite this intriguingly high number of oocytes, there is still a lack of explanation for physiological differences that account for the difference between *Bos indicus* and *Bos taurus* females in oocyte numbers. This lack of understanding has stimulated researchers to better understand folliculogenesis in Zebu females. Among the proposed hypotheses, a crucial aspect to be investigated is the population of preantral follicles. Despite suggestions that germline stem cells are present in the ovary [13,14], the bone marrow, or peripheral blood [15], preantral follicles constitute the non-replenishable pool of healthy follicles (ovarian reserve) that will be used throughout reproductive life.

There are approximately 2×10^6 germ cells in the ovaries of bovine fetuses at the end of the first trimester of pregnancy, but this number is drastically diminished during the last period of the fetal stage until birth [16] when follicular activation begins [17]. Follicles that are effectively recruited during the reproductive lifespan are those present at birth [18,19], but this ovarian reserve is highly variable [20–22]. The fate of ovarian follicles during postnatal life is ovulation or atresia; these two processes lead to a progressive reduction in the number of oocytes, as the reserve of preantral follicles is gradually consumed [20–23]. Thus, the population of preantral follicles represents the ovarian reserve, because it constitutes $>$ 90% of all ovarian follicles [24,25].

Despite valuable studies regarding follicular populations [26,27], little information is available regarding enumeration of preantral follicles in bovine females. A classic study stated that there were 2×10^5 primordial follicles per ovary [20]. However, studies on the total number of follicles in the ovaries of *Bos indicus* females are rare [28]. For instance, it is still to be determined whether the number of preantral follicles is a possible explanation for the greater number of oocytes that are obtained *in vivo* from *Bos indicus* females. Therefore, the aim of the present work was to compare the population of preantral follicles from *Bos taurus indicus* and *Bos taurus taurus* females by assessing the number of preantral follicles in ovaries of fetuses and heifers.

2. Materials and methods

2.1. Ovary collection

Ovaries (n = 64) were collected at abbatoirs from 180 to 240 d old *Bos taurus indicus* (Nelore, n = 10) and *Bos taurus taurus* (Aberdeen Angus, n = 10) fetuses, 20 to 24 mo old heifers (Nelore, n = 12, and Aberdeen Angus, n = 12) and 72 to 96 mo old cows (Nelore, n = 10, and Aberdeen Angus, n = 10). Fetal age was estimated following morphometric end points [29]. Ovaries from fetuses were collected from cows aged 150 to 210 mo. Heifers whose ovaries were collected had never been submitted to follicular aspiration throughout their reproductive lifespan. Both cows and heifers were kept on cultured pasture and fed mineral salt *ad libitum*. At slaughter, mean body condition of these animals was 4 ± 0.5 (scale, 1–5) [30]. All cattle were carefully evaluated according to body condition and health parameters before the death. Only ovaries without CL were used, to ensure good histological processing, and only one ovary per female was analyzed. 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 washed in tap water and placed in 70% alcohol.

2.2. Histological evaluation and follicle classification

Ovarian halves were dehydrated in alcohol, cleared with xylene, embedded in paraffin, and all the tissue was serially sectioned at 7 μm with a rotating microtome (Leica®, Wetzlar, Germany). In all ovaries, one of 120 histological sections [31] 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

Table 1
Mean (± SD) number of preantral follicles per ovary of *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) collected from fetuses (180–240 d), heifers (20–24 mo), and cows (72–96 mo).

Groups	No. preantral follicles per ovary			
	Primordial	Primary	Secondary	Total
Bos indicus fetuses (n = 10)	89,051 ± 40,050[ab]	53,454 ± 36,072[a]	1,423 ± 1,648[b]	143,929 ± 64,028[a]
Bos taurus fetuses (n = 10)	234,570 ± 310,058[a]	46,414 ± 17,772[a]	4,172 ± 3,437[a]	285,155 ± 325,195[a]
Bos indicus heifers (n = 12)	47,436 ± 61,888[c]	25,351 ± 19,926[b]	4,063 ± 2,891[a]	76,851 ± 78,605[bc]
Bos taurus heifers (n = 12)	83,726 ± 85,148[b]	21,010 ± 6,666[b]	4,937 ± 7,411[a]	109,673 ± 86,079[b]
Bos indicus cows (n = 10)	24,617 ± 22,057[c]	14,357 ± 10,728[b]	464 ± 312[c]	39,438 ± 31,017[c]
Bos taurus cows (n = 10)	64,395 ± 69,371[bc]	23,323 ± 18,073[b]	1,859 ± 1,477[ab]	89,577 ± 86,315[bc]

[a-c] Within a column, means without a common superscript differed ($P < 0.05$).

the developmental stage as 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 cuboidal granulosa cells) [32–33], and as normal or degenerated according to their morphological appearance. Follicles were considered degenerated if they had one or more of the following aspects: condensed oocyte nucleus, shrunken oocyte, pycnotic bodies in the granulosa cells, low cellular density, or basement membrane breakdown. Based on these parameters, only morphologically healthy follicles were evaluated [28]. Sections were examined and photographed using a light microscope (Nikon®, Tokyo, Japan). 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.

2.3. Follicular count

The number of preantral follicles was estimated by counting all follicles in each histological section; counting was done by only one operator in a blinded trial. To avoid counting the same follicle twice in the same section, a pen mark was made at the border of the histological section. Evaluation started from this point and followed a clockwise direction until the cortical portion had all been evaluated. 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 [34] and the following formula:

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

Nt = Estimated total number of follicle 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.

2.4. Statistical analysis

Results are presented as means ± SD. Bioestat 5.0 software [35] was used to test the normality of the samples, which was found not to be normal. Therefore, a Mann-Whitney test was used, and variables were evaluated two by two. Significance was set at $P \leq 0.05$.

3. Results

A total of 619 histological sections were analyzed from the 64 ovaries examined; on average, 1,203 follicles per section were counted. The average number of preantral follicles per ovary was similar between *Bos indicus* and *Bos taurus* females. There were (mean ± SD) 143,929 ± 64,028 preantral follicles in the ovaries of *Bos indicus* fetuses, 285,155 ± 325,195 in *Bos taurus* fetuses, 76,851 ± 78,605 in *Bos indicus* heifers, 109,673 ± 86,078 in *Bos taurus* heifers, 39,438 ± 31,017 in *Bos indicus* cows, and 89,577 ± 86,315 in *Bos taurus* cows (Table 1). There were differences between the average number of primordial follicles from *Bos indicus* (47,436 ± 61,888) and *Bos taurus* (83,726 ± 85,148) heifers ($P = 0.037$), and between the average number of secondary follicles, from *Bos indicus* (1,423 ± 1,648) and *Bos taurus* (4,172 ± 3,437) fetuses ($P = 0.041$), and from *Bos indicus* (464 ± 312) and *Bos taurus* (1,859 ± 1,477) cows ($P = 0.0006$; Table 1).

The number of preantral follicles varied among individuals within the same category and between breeds.

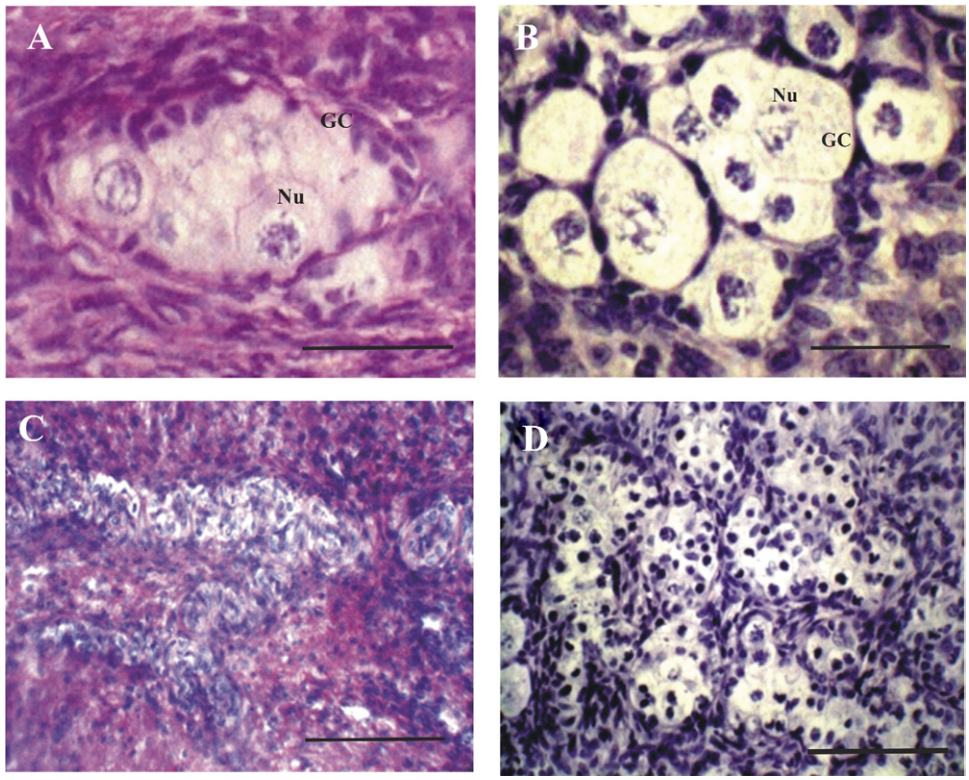

Fig. 1. Histological classification of polyovular follicles and cell cords. Polyovular follicles in Nelore (A) and Aberdeen Angus (B) heifers. Cell cords in the ovary from Nelore heifer (C) and Aberdeen Angus fetuses (D). Presumptive nucleus of oocytes (Nu) enclosed within a follicle-like cell with a single layer of granulosa cells (GC). Sections were stained with periodic acid Schiff (PAS) and hematoxylin. Bars = 50 μm. Original magnification X400.

Variation within Nelore fetuses ranged from 41,957 to 248,865 preantral follicles, and from 50,326 to 1,090,140 in Angus fetuses. Variation in the ovaries of heifers ranged from 9,623 to 260,371 preantral follicles per ovary in Nelore and from 33,798 to 320,729 follicles in Angus, and from 8,010 to 94,301 in the ovaries of Nelore cows, and from 10,043 to 253,453 preantral follicles in Angus cows.

Among the ovaries assessed, 26 of 64 (41%) had polyovular follicles (both primordial and primary). Polyovular follicles were observed in the ovaries of Nelore fetuses (n = 2), heifers (n = 3) and cows (n = 3), and in those of Angus fetuses (n = 6), heifers (n = 5), and cows (n = 6). The number of oocytes within a polyovular follicle ranged from 2 to 9 (Fig. 1). The follicular population in the ovaries with polyovular follicles (n = 26) ranged from 25,166 (Nelore cow) to 1,090,140 (Angus fetus), and half of them (n = 13) had a larger population of ovarian follicles than the category average. Cell cords were also observed in the ovaries of two Nelore heifers, one Angus fetus and one Angus heifer. These structures were not observed in any ovary from Nelore and Angus cows. There was no apparent association between the presence of cell cords and the number of follicles per ovary. The average number of preantral follicles in the ovaries with cell cords (36,789 and 182,189 in Nelore heifers, 121,529 in Angus fetus and 91,673 in Angus heifer) was smaller than the average follicular population.

4. Discussion

Herein, we reported the first comparative study of the population of preantral follicles in ovaries from *Bos indicus* (Nelore) and *Bos taurus* (Angus) fetuses heifers and cows and contributed with new information regarding the number and morphology of preantral follicles in these bovine breeds.

The follicular population (143,929 follicles) detected in the ovaries of Nelore fetuses was close to a previous report (163,216 follicles) in 180 d fetuses [36]. However, our findings (285,155 follicles) in Angus fetuses were higher than the approximately 102,000

follicles reported for Holstein fetuses at the end of pregnancy [16]. There are conflicting opinions on the number of follicles in the fetal period; some authors suggest significant variation according to the stage of pregnancy [16,36], whereas others indicate the opposite [33], particularly after the fifth month of pregnancy. Despite this controversy, our study was performed with fetal ovaries collected between 6 and 8 mo of gestation, which is considered a period in which there is less variation in the population of preantral follicles [37].

The average number of preantral follicles (109,673) in *Bos taurus taurus* heifers was slightly less than that described originally [20] (132,000), for 14 *Bos taurus* heifers (Hereford) aged 19 to 24 mo. The difference of approximately 20,000 follicles can be considered small, since hundreds of thousands of structures are present in the ovary. Considering the similar number of animals and the age groups studied, our results agreed with those from Erickson [20], with an estimate of approximately 120,000 preantral follicles for *Bos taurus* heifers up to the age of 24 mo old. Regarding the population of preantral follicles in *Bos taurus indicus* cows, other authors [28] reported an average of 70,576 follicles per ovary from Nelore cows, which was higher than the 39,438 follicles observed in our work. Similarly, the number of follicles in the ovaries from Angus cows presented in this study (89,577) seemed to be higher than that previously detected in 16 Hereford cows aging 84 to 108 mo (22,000) [20].

The population of follicles in ovaries is remarkably variable at birth. Consequently, adults also have a highly variable ovarian reserve throughout their reproductive lifespan [20,21]. This high individual variation makes it difficult to apply any statistical model to ovarian follicle number. Reports of variation in the number of follicles among animals include extremes from 0 to 700,000 [20], and similar situations were observed by others [16]. Our findings were consistent with these previous reports, given the variation in the number of follicles in both *Bos indicus* (from 41,958 to 248,865) and *Bos taurus* (from 50,326 to 1,090,140) fetuses. There was also large variation for heifers in the present study, with follicular populations between 9,623 and 260,371 in *Bos indicus* and between 33,798 and 320,729 in *Bos taurus*.

This high individual variation in the number of ovarian follicles seemed to be constant throughout the reproductive lifespan, since there is high reproducibility in the number of follicles per wave in adulthood [38]. In line with these data, were previous observations from studies collecting oocytes *in vivo* using OPU [2]. Variation in the number of preantral follicles might be an indicator of why some animals can yield numerous oocytes in a single OPU procedure. In zebu females, recent studies suggested that the individual variation in the numbers of oocytes obtained from OPU was correlated with the expression of GDF9, BMP15, and FGF8 genes [39]. These authors reported an increase of 2.26 ± 1.08 oocytes, considering only the effect of FGF8, as well as a possible increase of 7.36 ± 1.12 oocytes when all genes were considered together. In addition to the promising aspects described [39], other factors and/or genes may be involved, since variations among individual animals seemed much higher than those reported by these authors.

Bos indicus females have more follicular waves [4] and more follicles per wave [5] than *Bos taurus* females. Furthermore, there is high repeatability in the number of follicles per wave [38]. If *Bos indicus* females do not have more preantral follicles, perhaps they have lower rates of follicular atresia compared to *Bos taurus*, resulting in more viable antral follicles. Indeed, depletion of follicular reserves is associated with high rates of follicular atresia [40]. Yet, whether or not *Bos taurus* females have higher rates of follicular atresia compared to *Bos indicus* females remains to be determined.

In the present study, polyovular follicles were present mainly in adult ovaries. Among the total number of ovaries evaluated, polyovular follicles were observed in 41% (26/64) of ovaries (Fig. 1). Polyovular follicles are follicles in which two or more oocytes are contained within a single follicle without a separating basement membrane between them [41]. They can contain up to 24 oocytes [42,43]. For Nelore females, such structures have already been described in cows with a 83% (5/6) frequency [28], and in fetuses during the first trimester of pregnancy, which corresponds to the period of oogenesis, as "nests of oogonia" [36]. Polyovular follicles were also reported in antral or preantral follicles from ovaries of *Bos taurus* crossbred heifers [44]. In early fetal life, such structures have also been described in humans [45] and named "cysts" [46]. Thus, it is intriguing to observe such structures during the late fetal period and especially in heifers, as we have shown in both *Bos taurus* and *Bos indicus* females [28].

In addition to polyovular follicles, we also observed cell cords in Nelore heifers and Angus heifers and fetuses (Fig. 1), which were similar to the ovarian cords described for germ cells and pregranulosa cells. While germ cells are dividing to form cysts, they also interact with epithelial pre-granulosa cells, becoming organized into ovigerous or ovarian cords, which remain until primordial follicles begin to form [47–50]. The occur-

rence of polyovular follicles and ovarian cords was reported during fetal life [36,51], but their biological significance remains to be determined. Germ cell clusters appear in greater quantity at the beginning of fetal life and gradually disappear as more primordial follicles are formed. At the end of fetal life, ovarian cords have almost completely disappeared, and secondary follicles are present [51]. Both polyovular follicles and cell cords reported in the present study could represent follicular renewal activity, since these structures are typically described in early folliculogenesis. However, more accurate methods are needed for further investigations. It was noteworthy that there was no association among these structures and the number of ovarian preantral follicles. However, recent studies reported that the high variation in the number of antral follicles among *Bos taurus* heifers was highly correlated with the number of polyovular follicles [44].

In summary, the present results reported will provide the basis for further studies to improve understanding of factors regulating folliculogenesis in cattle. There was substantial variation among individual animals, but the overall population of preantral follicles did not differ significantly among *Bos indicus* and *Bos taurus* fetuses and heifers. Therefore, we inferred that the greater numbers of oocytes produced by *Bos indicus* compared to *Bos taurus* females in ovum pick-up programs were not due to the number of preantral follicles in the fetal or postnatal female.

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