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Cell proliferation in ovarian follicles from *Bos taurus indicus* females with different antral follicle count

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Abstract

The aim of this study was to compare the cell proliferation rate of follicles from *Bos taurus indicus* females with different antral follicle counts (AFCs). Thirty pairs of ovaries were classified as having low (≤ 31 follicles), intermediate (≥ 46 and ≤ 76 follicles) or high AFC (≥ 91 follicles). The ovaries were cut into 1 x 1 x 0.3 cm fragments, fixed in 10% buffered formalin solution and subjected to immunohistochemical analysis for proliferating cell nuclear antigen (PCNA). PCNA detection was detected in all stages of follicular development, being 1,388 (82.23%) primordial, 197 (11.67%) primary, 29 (1.72%) secondary and 74 (4.83%) antral follicles. The PCNA detection rate was higher ($P \leq 0.05$) for antral and secondary follicles in ovaries with intermediate and low AFCs. This study showed that higher PCNA at early stages of follicular growth is detected in animals with high AFC, whereas a higher PCNA is seen in secondary and antral follicles of animals with low AFC.

Keywords: bovine, follicular development, immunohistochemistry, ovaries, PCNA.

Introduction

The antral follicle count (AFC) is a remarkable reproductive feature that can be determined quickly and accurately. Therefore, after an ultrasound examination, females can be classified into groups defined in number by a low, intermediate or high antral follicles Some studies have been proposed to elucidate the influence of AFC on the fertility of cows because the number of antral follicles in the same animal is highly repeatable (Burns *et al*., 2005; Ireland *et al*., 2007, 2008, 2009; Silva-Santos *et al*., 2014). The AFC in *Bos taurus* is directly correlated with the ovarian follicular reserve (Ireland *et al*., 2011), but this relationship has not been fully elucidated in *Bos taurus indicus* females. *Bos taurus indicus* females have a higher population of antral follicles than *Bos taurus* (Batista *et al*., 2014), generating a greater number of viable oocytes (Pontes *et al*., 2011).

Mossa *et al*. (2012) reported that *Bos taurus* cows with low AFCs have lower fertility than those with high AFCs. In addition, they reported a positive correlation between high AFC, and better reproductive efficiency in several aspects studied. However, recent studies have revealed different patterns in Zebu cattle. When comparing pregnancy rates after artificial insemination, *Bos taurus indicus* cows with low AFCs presented better results, while females with high AFCs showed lower pregnancy rates (Santos *et al*., 2012, 2013; Morotti *et al*., 2014; Santos *et al*., 2016).

Considering the contrast between practical aspects, such as pregnancy rates, described in those reports, it would be interesting to evaluate the physiological mechanisms of ovarian activity according to the AFC. Proliferating cell nuclear antigen (PCNA) is a protein that plays an essential role in regulating cellular functions of eukaryotic organisms (Strzalka *et al*., 2015). It is well established that the PCNA is a marker for cellular proliferation and is associated with DNA synthesis in all eukaryotic species that include *Bos taurus indicus* (Strzalka and Ziemienowicz, 2007; Sun *et al*., 2012). In this way, the investigation of PCNA activity on follicular activity in cattle ovaries should contribute to clarify the controversial aspects mentioned above, mainly in *Bos indicus* females.

Therefore, the aim of this study was to evaluate the cell proliferation of follicles from *Bos taurus indicus* females with a low, intermediate or high antral follicle count.

Material and methods

Ovaries

Ovaries (n = 60) from cyclic (presence of corpus luteum) Nelore (*Bos taurus indicus*) cows (n = 30), 72 to 96 months of age, with a body condition score (BCS) from 4 to 4.5 (scale of 1 to 5; Lowman *et al*., 1976) were obtained from a local slaughterhouse (latitude 23° 17' 34" S and longitude 51° 10' 24" W), and transported to the laboratory within 20 minutes. The ovaries were placed in pairs, separated by animal, and transported in an insulated box with NaCl 0.9% solution at 30°C. At the laboratory, each pair of ovaries was placed in a container with NaCl 0.9% solution, and the AFC was assessed by the same operator using a real-time B-mode ultrasound scanner (Scanner 200 Vet; Pie Medical, Maastricht, The Netherlands). All antral follicles (≥ 2 mm in diameter) were identified and counted. Considering the AFC average and standard deviations, each pair of ovaries was classified into one of three groups: low (≤ 31 follicles, n = 6), intermediate

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(≥ 46 and ≤ 76 follicles, n = 16) and high AFC (≥ 91 follicles, n = 8).

Histological processing

The AFC were classified in categories. Fifteen pairs of ovaries were designated for histological processing (n = 5 ovaries for each AFC group). We excluded those pairs of ovaries (n = 15) whose AFC did not meet the criteria described above. Only one ovary from each pair was selected according to the absence of a corpus luteum and/or pre-ovulatory follicle. The selected ovaries were cut into fragments approximately 1 x 1 x 0.3 cm, and then fixed in 10% buffered formalin solution for 8 hours. Subsequently, these fragments remained in 70% alcohol for 24 hours, after being dehydrated in increasing concentrations of alcohol, diaphanized in xylene, and embedded in paraffin blocks included in histological sections. The serial sections, 5-µm thick, were cut on the microtome (Leica®, Wetzlar-Germany). The slides were processed for staining with hematoxylin and PAS. The best fragments were designed for immunohistochemical assessment of proliferative activity using PCNA.

Immunohistochemistry

Cell proliferation was evaluated by immunohistochemical staining technique using the detection of PCNA. We used the mouse anti-PCNA (monoclonal antibody clone PC1O, Novex®, 1:100 dilutions) primary antibody and anti-mouse goat (Invitrogen, 1:100 dilution) secondary antibody. The positive control was skin cancer and negative control was performed with the absence of primary antibody. Both positive and negative controls were used according to the manufacturer's instructions, following the same dilutions used in the experimental samples.

Histological sections were deparaffinized, rehydrated and subjected to antigenic recovery in tris-EDTA Tween solution (pH 9.0) with incubation in an electric pressure cooker for about nine minutes. Subsequently, the sections were subjected to blocking of endogenous peroxidase with 10 volumes of hydrogen peroxide (3%) for 30 minutes, and blocking of nonspecific reaction with the membrane blocking solution (Invitrogen TM) for 30 minutes. Thus, they were incubated with primary antibody anti-PCNA overnight (18 hours) at 4°C on a rocker. After incubation, the secondary antibody was applied for 30 minutes, and then the reaction was revealed using diaminobenzidine substrate (DAB) in a brown color in approximately 5 minutes. Subsequently, the material was stained with Harris Hematoxylin, dehydrated, and the slides were mounted to analyzed under a light microscope (Nikon®, Tokyo, Japan).

Follicles were classified according to the stage of development, considering the shape, and layers of the granulosa cells (GC), as follows: primordial (oocyte surrounded by a flat granulosa cell layer); primary (one layer of cuboidal granulosa cells); secondary (two or more layers of cuboidal cells); or antral (three or more layers of cuboidal granulosa cells and the presence of antrum) according Silva-Buttkus *et al*. (2008).

Statistical analysis

The mean number and standard deviation (SD) were extracted from the total population of antral follicles from all 60 ovaries. The low AFC group was determined based on the population mean≈(6 1 follicles) minus the SD (≈ 30 follicles) (Low AFC≤ 31 follicles; n = 6). The intermediate group was within the range of≥ 46 and ≤ 76 follicles (n = 16). The high AFC group was based on the population mean plus the SD value (High AFC≥ 9 1 follicles; n = 8). The data were presented as proportions for the descriptive statistical analyses. The Fisher's exact test was used to evaluate the difference in the percentage of immunostained follicles among AFC groups (high, intermediate and low count). The statistical analyses were performed using Minitab® statistical 16.1.1 software, and a p value ≤ 0.05 indicated a significant difference.

Results

A total of 1,688 follicles were evaluated by staining of PCNA in *Bos taurus indicus* females with low, intermediate and high AFCs. We classified 1,388 (82.23%) follicles as primordial, 197 (11.67%) as primary, 29 (1.72%) as secondary and 74 (4.83%) as antral follicles (Table 1). Considering the comparison among the AFC groups (low *versus* intermediate *versus* high), primordial follicles from cows with a high AFC showed a higher (P ≤ 0.05) PCNA detection rate compared to the intermediate group. However, secondary and antral follicles had higher (P ≤ 0.05) PCNA detection rates in the groups with intermediate and low AFCs.

Table 1. Percentage of follicles stained for proliferating cell nuclear antigen from bovine females with high (AFC ≥ 91 follicles), intermediate (≥ 46 and ≤ 76 follicles) and low antral follicle counts (≤ 31 follicles).

Follicles	High % (n)	Intermediate % (n)	Low % (n)	Total % (n)
Primordial	85.60[a] (1,177)	63.09[b] (94)	71.34[ab] (117)	82.23 (1,388)
Primary	11.13[a] (153)	15.44[a] (23)	12.80[a] (21)	11.67 (197)
Secondary	1.16[b] (16)	4.70[a] (7)	3.66[a] (6)	1.72 (29)
Antral	2.11[b] (29)	16.75[a] (25)	12.20[a] (20)	4.83 (74)
Total	1375	149	164	1688

Values withdifferent superscripted letters within the same rows (comparing AFC groups) indicate statistical difference (P ≤ 0.05).

The PCNA detection observed in primordial and primary follicles was restricted to the oocyte. The PCNA signal in secondary and antral follicles was identified in the oocyte as well as in the granulosa and theca. In follicles with only a few layers of granulosa and a small number (<10) of stained cells, there was no staining on theca cells. On the other hand, in those follicles with more layers of granulosa and theca cells, we easily found stained cells in the theca layers, a situation also observed in tertiary and large antral follicles (Fig. 1).

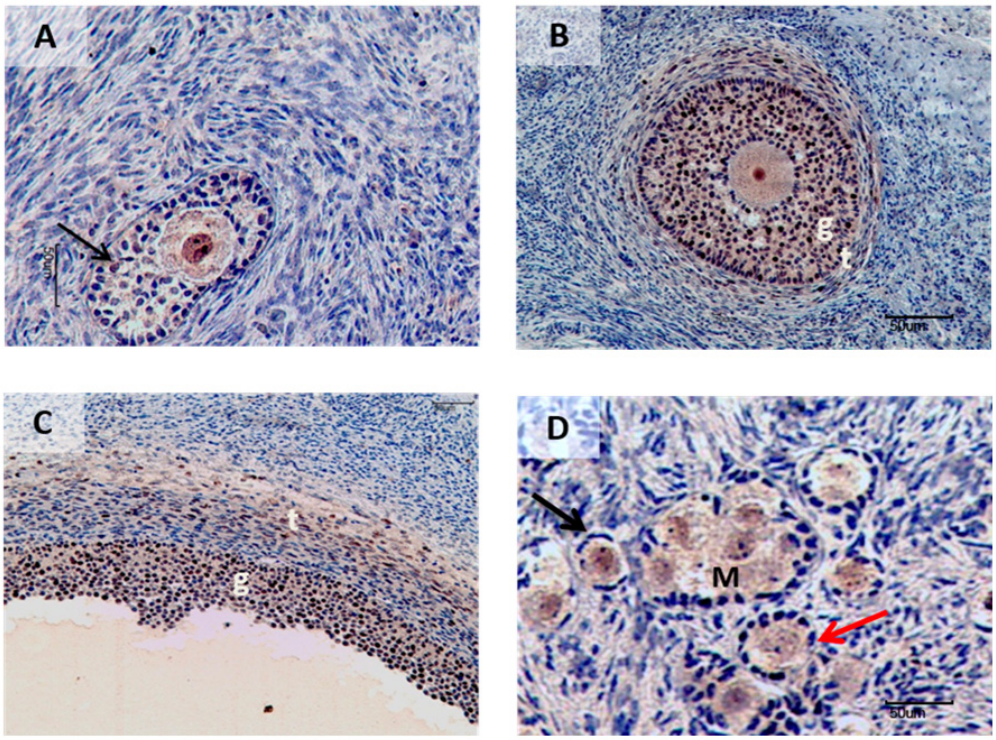

Figure 1. Ovarian follicles of *Bos taurus indicus* females marked in brown color for the proliferating cell nuclear antigen. A – Detection of proliferating cell nuclear antigen in nucleus, cytoplasm and granulosa cells (arrow) in initial secondary follicle. B – Detection of proliferating cell nuclear antigen in nucleus, cytoplasm, granulosa cells (g) theca (t) in secondary follicle. C - Layers of granulosa (g) and theca (t) of antral follicle. D – Detection of proliferating cell nuclear antigen in nucleus and cytoplasm of primordial follicles (black arrow), primary (red arrow), and detection of proliferating cell nuclear antigen in multioocytes (M).

Discussion

The follicular cell proliferation was evaluated using the anti-proliferating cell nuclear antigen antibody according to the antral follicle population in cattle. We identified a higher proliferative activity in the final stages of follicular development from females with intermediate and low AFCs.

The present study is based on the principle that each cow can be classified according to the number of antral follicles in the ovaries. In performing just one ultrasound examination, there is a high repeatability for classifying females as having a low, intermediate or high AFC (Ireland *et al*., 2007, 2008; Mossa *et al*., 2010; Silva-Santos *et al*., 2014).

In studies carried out with *Bos taurus indicus* females, a positive correlation between high AFC and better reproductive efficiency was described (Ireland *et al*., 2007; 2008; Evans *et al*., 2012), and a low AFC was associated with poor fertility (Mossa *et al*., 2012). However, several studies performed in *Bos taurus indicus* cattle submitted to fixed time artificial insemination (FTAI) showed better pregnancy rates in females with a low AFC (Santos *et al*., 2013; Morotti *et al*., 2014; Santos *et al*., 2016). Interestingly, there is a larger follicular diameter in females with low AFC (Santos *et al*., 2012; Morotti *et al*., 2016). This information is relevant because larger ovulatory follicles have been associated with better conception rates in FTAI programs (Perry, 2007; Meneghetti *et al*., 2009). Our results on cell proliferation indicated a more intense follicular activity in cows with a low AFC, at least for follicles at the final stages of development. This finding is in accordance with those aspects mentioned above, regarding better rates of pregnancy for *Bos taurus indicus* cows with a low AFC.

In the present study, theca cells of secondary and antral follicles showed staining for PCNA, which is in accordance with previous studies that demonstrated a PCNA signal in different stages of follicular growth, mainly after the primordial stage (Oktay *et al*., 1995; Wandji *et al*., 1996; Tománek and Chronowska, 2006). The PCNA staining for proliferative activity is closely related to cellular activity for DNA repair, and cell division (Myoung *et al*., 2006; Strzalka and Ziemienowicz, 2011).

Comparing the same follicular category among AFC groups, the present study showed that animals with

intermediate or low AFCs showed more intense staining indicative of cell proliferation in the secondary, and antral follicular stages than those with a high AFC. On the other hand, animals with a high AFC showed a higher cell proliferation rate in the early stages of follicular growth. Therefore, we identified a distinct pattern of cell proliferation in the follicular stages that varies according to the AFC.

A clear connection between AFC and fertility remains contradictory in cattle. Considering Zebu cattle, some articles presented data favoring low AFC (Santos *et al*., 2013; Morotti *et al*., 2014; Santos *et al*., 2016). However, others teams showed best fertility rates for high AFC in European breeds (Ireland *et al*., 2007; 2008; Evans *et al*., 2012). The majority of the articles focused only on pregnancy rates, hormonal dosage or embryo production and not on follicular activity. We believe that our data with PCNA activity are bringing an important novelty for a better comprehension on the matter, mainly considering the distinct pattern of staining we described in the results.

In conclusion, the cell proliferation evaluated by immunostaining for proliferating cell nuclear antigen differs between follicular categories, with a higher proliferation rate in groups with intermediate and low antral follicle counts.

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