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Expression of androgen-producing enzyme genes and testosterone concentration in Angus and Nellore heifers with high and low ovarian follicle count

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ABSTRACT

Follicle population is important when animals are used in assisted reproductive programs. *Bos indicus* animals have more follicles per follicular wave than *Bos taurus* animals. On the other hand, *B taurus* animals present better fertility when compared with *B indicus* animals. Androgens are positively related with the number of antral follicles; moreover, they increase growth factor expression in granulose cells and oocytes. Experimentation was designed to compare testosterone concentration in plasma, and follicular fluid and androgen enzymes mRNA expression (*CYP11A1*, *CYP17A1*, *3BHSD*, and *17BHSD*) in follicles from Angus and Nellore heifers. Heifers were assigned into two groups according to the number of follicles: low and high follicle count groups. Increased testosterone concentration was measured in both plasma and follicular fluid of Angus heifers. However, there was no difference within groups. Expression of *CYP11A1* gene was higher in follicles from Angus heifers; however, there was no difference within groups. Expression of *CYP17A1*, *3BHSD*, and *17BHSD* genes was higher in follicles from Nellore heifers, and expression of *CYP17A1* and *3BHSD* genes was also higher in HFC groups from both breeds. It was found that Nellore heifers have more antral follicles than Angus heifers. Testosterone concentration was higher in Angus heifers; this increase could be associated with the increased mRNA expression of *CYP11A1*. Increased expression of androgen-producing enzyme genes (*CYP17A1*, *3BHSD*, and *17BHSD*) was detected in Nellore heifers. It can be suggested that testosterone is acting through different mechanisms to increase follicle development in Nellore and improve fertility in Angus heifers.

1. Introduction

It is well known that *Bos indicus* and *Bos taurus* animals behave differently when referring to reproduction [1]. Taurine animals reach sexual maturity earlier [2,3] and have smaller calving intervals than indicine females [4]. Moreover, their estrus is more evident and last for more hours [5]. On the other hand, *B indicus* animals develop considerably more follicles per follicular wave than *B taurus* animals [6,7]. This feature is especially important for animals that are used in assisted reproductive technologies such as superovulation, ovum pick-up, and *in vitro* embryo production.

The estrus cycle and follicular development are stimulated by hormones such as follicle-stimulating hormone (FSH), luteinizing hormones, and estrogen. Although in reduced concentrations, androgens are also important to promote follicular growth. They are synthesized by the thecal cells under the influence of luteinizing hormone

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through the enzymatic conversion of androgen precursors [8]. In the follicular fluid, androgens can act by increasing FSH actions, or serve as a substrate to the production of estradiol by aromatase [9].

In bovines, androgen concentrations are positively related with the number of antral follicles in the ovary [10]. Furthermore, treatment with androgens increased the expression of growth factors in granulosa cells and oocytes of monkeys [11] and mice [8,12]. *In vitro*, treatment of preantral follicles with androgen increased follicle development and decreased apoptosis [13]. In addition, culture of granulosa cells with testosterone decrease mRNA and protein levels of anti-Mullerian hormone, a hormone that is known to hinder follicle development [14].

The present study aimed to evaluate expression of androgen-producing enzyme genes in antral follicles collected from Angus and Nellore heifers with low and high ovarian follicle count and measure plasma and intrafollicular testosterone concentration in these animals.

2. Materials and methods

All reagents and media used were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. Animals were housed and cared for in accordance with the guidelines described by the ethics committee of the Universidade do Estado de São Paulo (Botucatu).

2.1. Animal selection

To pre-select the animals an ultrasound (US) examination (Mindray, 5–10 MHz, China) was performed in a random day of the estrus cycle in 100 Angus and 100 Nellore heifers. Only heifers that were cycling and did not have follicles greater than 5 mm were selected. In this first evaluation, the total number of follicles in both ovaries was counted and the mean follicle number was determined for each breed. These heifers were synchronized with two doses of PGF2α 11 days apart. Four days after the second PGF2α (approximately 24 hours after follicle recruitment), a second US evaluation was performed to confirm the total number of follicles in each heifer. Considering the mean ± standard deviation (SD) of the follicle population in each breed, the heifers were classified into two groups as follows: the low follicle count group (LFC; heifers that had the total number of follicles below the mean minus the SD) and high follicle count group (HFC; heifers that had the total number of follicles above the mean plus the SD). A total of 18 Nelore heifers were selected and allocated into two groups: eight with HFC (≥40 follicles) and 10 with LHC (≤20 follicles). For Angus heifers, 22 were selected and allocated into two groups: 13 with HFC (≥20 follicles) and 09 with LFC (≤13 follicles).

Heifers were kept in *Brachiaria brizantha* grass *ad libitum*, were fed 2 kg of *Cynodon* spp hay and 4 kg of concentrate (16% crude protein and 70% total digestible nutrients)/per animal/day during 90 days. After this adaptation period, all heifers were yet again synchronized using the same protocol (two doses of PGF2α 11 days apart). When heifers showed estrus, they were evaluated by US every 12 hours until ovulation. Twenty-four hours after ovulation, heifers were slaughtered in a local abattoir.

After slaughter, ovaries were collected and placed in a saline solution at 4 °C to be transported to the laboratory. Three follicles from 2 to 4 mm in diameter were dissected from the ovary contralateral to the corpus luteum. Follicles were frozen individually in 1-mL Qiazol from the RNeasy Microarray Tissue Mini kit (Qiagen, Valencia, CA, USA) at −80 °C.

2.2. Real-time polymerase chain reaction

This experiment was designed to evaluate the mRNA expression of androgen-producing enzymes. Twenty-two Angus heifers (LFC = 09 and HFC = 13) and 18 Nellore heifers (LFC = 10 and HFC = 08) were used in this experiment (these heifers were selected out of the 200 heifers US at the beginning). Total RNA was extracted individually from three follicles from each animal using the RNeasy Microarray Tissue Mini kit following the manufacturer's instructions. Follicles were homogenized with an Ultra-Turrax (IKA, Campinas, SP, Brazil) for 1 minute in 1 mL of QIAzol. Total extracted RNA was stored at −80 °C until real-time polymerase chain reaction analysis. RNA samples (1 μg) were incubated with DNAse I (1 U/μg; Invitrogen) and reverse transcribed with SuperScript III (Invitrogen) and oligo-dT primers.

Quantitative real-time polymerase chain reaction analysis of four androgen-producing enzyme genes (Cholesterol side chain cleavage enzyme [*CYP11A1*], 17-alpha-hydroxylase [*CYP17A1*], 3-beta-hydroxysteroid dehydrogenase [*3BHSD*], and 17-beta-hydroxysteroid dehydrogenase [*17BHSD*]) and a housekeeping gene (peptidylprolyl isomerase A; *PPIA*) was performed on each follicle. Specific primers (Table 1) were designed using Integrated DNA Technologies software (http://idtdna.com). To select the most stable housekeeping gene for detailed analysis of each cell type, *PPIA*, glyceraldehyde-3-phosphate dehydrogenase, and histone H2AFZ (*H2AFZ*) amplification profiles were compared using the geNorm applet for Microsoft Excel (http://genorm.cmgg.be) [15]. On the basis of this comparison, the relative quantification was performed with *PPIA*. Power SybrGreen PCR Master Mix (Applied Biosystems) reaction chemistry and the ABI Prism 7500 Sequence Detection System (Applied Biosystems) were used to quantify mRNA concentrations, and the specificity of each polymerase chain reaction product was determined through melting curve analysis. Negative controls (in which water replaced complementary DNA) were run in each plate. Duplicate reactions of each sample were analyzed. Target gene mRNA abundance is expressed relative to the level of *PPIA* mRNA. The relative expression of each gene was determined using the ΔΔCt method. Results are expressed as fold change ($2^{-\Delta\Delta CT}$).

2.3. Plasma and follicular fluid testosterone concentration

Blood for the testosterone was drawn on the day of slaughter (24 hours after ovulation), centrifuged for 10 minutes at 900 × *g*, and plasma was frozen at −80 °C. The follicular fluid was collected from three 2 to 5 mm follicles