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Serum profile of the anti-müllerian hormone of Nelore bulls in the peripuberty

[*Perfil sérico do hormônio anti-Mülleriano de touros Nelore na peripuberdade*]

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ABSTRACT

This study aimed to investigate the profile of AMH serum concentrations during the period of prepuberty in young bulls. The bulls were selected and evaluated for 150 days to assess their sexual development. The semen and blood collection, body weight (kg), and scrotal measurements were performed every 30 days, and puberty (D0) was considered when at least 50×10^6 spermatozoa/mL, and 10% of progressive motility were verified for the first time. After the blood collection, plasma was separated and used to determine the AMH concentration by ELISA, presenting values of 611.4a (±94.5), 555a (±181.99), 621.6ab (±133.44), 370.4ab (±59.36) and 51.7b (±7.94) for moments of -60, -30, 0, +30 and +60 of puberty, respectively. During the evaluated period, there was a progressive increase in body weight (kg), scrotal circumference (cm), and semen characteristics. The AMH mean plasma concentration decreased 60 days after puberty onset. There was no correlation with the andrological parameters analyzed; however, there was a positive and strong correlation between the testicular height and width at puberty onset with plasma concentrations of AMH. We concluded that AMH is not a helpful tool for the early prediction of puberty in Nelore bulls.

Keywords: AMH, *Bos indicus*, puberty, younger bulls

RESUMO

Este estudo teve como objetivo investigar o perfil das concentrações séricas do AMH no período da peripuberdade em tourinhos. Oito bezerros da raça Nelore, com oito meses de idade, foram incluídos neste estudo. Os touros foram selecionados e avaliados por 150 dias para se avaliar seu desenvolvimento sexual. A coleta de sêmen e sangue, o peso corporal (kg) e as medidas escrotais foram realizadas a cada 30 dias, e a puberdade (D0) foi considerada quando pelo menos 50×10^6 espermatozoides/mL e 10% de motilidade progressiva fossem verificadas pela primeira vez. Após a coleta de sangue, o plasma foi separado e utilizado para determinação da concentração de AMH por ELISA, apresentando valores de 611,4a (±94,5), 555a (±181,99), 621,6ab (±133,44), 370,4ab (±59,36) e 51,7b (±7,94) para os momentos de -60, -30, 0, +30 e +60 da puberdade, respectivamente. No período avaliado, houve aumento progressivo do peso corporal (kg), da circunferência escrotal (cm) e alterações nas características do sêmen. A concentração plasmática média de AMH diminuiu 60 dias após o início da puberdade. Não houve correlação com os parâmetros andrológicos analisados; no entanto, houve correlação positiva e forte entre a altura e a largura testicular no início da puberdade com as concentrações plasmáticas do AMH. Concluiu-se que o AMH não é uma ferramenta útil para a predição precoce da puberdade em touros Nelore.
Palavras-chave: AMH, Bos indicus, puberdade, touros precoces

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INTRODUCTION

Anti-müllerian hormone (AMH) is a dimeric glycoprotein, and it is considered a member of the transforming growth factor β (TGF-β) superfamily. This glycoprotein is responsible for promoting the degeneration of the Müller ducts during sexual embryonic differentiation (Cate *et al*., 1986), its expression is triggered by SOX9 in Sertoli cells at the onset of testicular differentiation, and regulated by SF1, GATA factors, WT1, DAX1 and FSH (Rey *et al*., 2003; Xu *et al*., 2019).

Embryonic Sertoli cells can produce high levels of AMH from sexual differentiation to puberty, decreasing after this period (Rajak *et al.*, 2017), as confirmed by Rey *et al*. (2003), in review, were assertive in claiming that, except for a transient decline in the peri-Natal period, the testicular AMH secretion is maintained at high levels to puberty, when the ripening of Sertoli cells is characterized by a Decreasing AMH activity, which would enable its use to determine the maturation of Sertoli cells.

The AMH abundance in the testicular tissue is increased at the first month of age and decreases significantly as age advances, being low at 24 months (Rico *et al*., 2011). Studies have indicated that testicular AMH expression remains high until puberty in several cattle breeds (Rajak *et al*., 2017; Rota *et al*., 2002). However, blood concentrations of AMH decrease as Sertoli cells reach maturity (Josso, 1992). According to Rey *et al*. (2003), AMH's secretion behaves differently between the pre and pos pubertal periods, being secreted through the basal membrane into the interstitial tissue to reach the bloodstream, before puberty and then, when occluding junctions are established between Sertoli cells during puberty, AMH secretion is directed mainly to the lumen of the seminiferous tubules. Studies with Simental and Brahman bulls (Maculan *et al*., 2018) and stallions (Almeida *et al*., 2012), suggested that the measurement of AMH concentration can be used as a biological marker to identify early puberty.

This study aimed to determine the serum values of AMH with sperm patterns during the peripubertal phase, assessing 60 days before puberty to 60 days after the diagnosis of puberty.

MATERIAL AND METHODS

The study was conducted following the recommendation of the National Council of Animal Control and Experimentation (CONCEA) and was approved by the Institutional Committee on the Ethics in Use of the Animal (protocol #09/2021). The study was carried out in the North Brazilian region (9º 11' 33" S and 68º 47' 37" W), which has a humid climate and sub-humid equatorial.

For the proposal, 8 Nelore calves (*Bos indicus*) aged 8 months, all contemporaries, with mean ± standard deviation weight of 245.2 ± 12.1 kg were selected from a herd (n = 30). The animals were kept in a grazing system in a paddock with forage cultivated from *Brachiaria humidicola*, mineral supplementation (Techsal®, Socil, Paulínia, São Paulo, Brazil), 1% body-weight creep feeding, and water *ad libitum.*

In a herd of 30 calves, 8 were selected for the study according to Wolf' *et al.* (1965) criteria. Initial puberty (D0) was considered when the animals presented at least 50×10^6 spermatozoa/mL and 10% of progressive motility for the first time. The calves were assessed for 150 days, every 30 days from D-60 to D+60 (8, 9, 10, 11, and 12 months), according to Brito *et al*. (2004). The animals were contained in a stock containing a digital scale to be weighed (kg). The scrotal circumference (cm) measurement according to the American Society for Theriogenology guidelines, 1976, adapted by Chenoweth and Ball (1980).

The semen was obtained by electroejaculation. The animals were restrained in a chute, the rectum was emptied, and the entire lubricated probe was inserted rectally with the electrodes oriented ventrally. Then, electrical pulses (voltage ranging from 8 to 16 V) were released for 1–2 seconds with a period of rest of 0.5–2 seconds until the occurrence of ejaculation. During stimulation the voltage of pulses gradually increased (Palmer *et al*., 2005). The ejaculate was assessed for volume, subjective motility and vigor, sperm concentration, and morphology (Henry and Neves, 2013).

The volume was evaluated in a graduated tube, and a drop of semen was placed on a heated slide

covered by a coverslip. The motility (%) and vigor (score 1 - 5) were assessed in phase-contrast microscopy (100X magnification; Eclipse E200 microscope®). Then, the semen was diluted (1:50) in a formol-saline solution to determine sperm concentration (x10 spermatozoa/mL) in a Neubauer chamber. The sperm morphology was evaluated using a semen aliquot diluted in formol-saline to determine the percentage of normal spermatozoa or with major or minor defects (Barth and Oko, 1989; Henry and Neves, 2013).

The blood samples were collected from the jugular vein using 10 mL vacuum tubes containing heparin (BD Vacutainer®, Curitiba, Paraná, Brazil). The plasma was obtained by centrifugation (2,100 g for 25 minutes, at 10 ºC) and stored at -20 ºC. The determination of the AMH was performed in duplicates, using an ELISA kit (Bovine AMH ELISA - AL-114, Ansh Labs, Webster, TX, USA), following the manufacturer's instructions and a standard recombinant human AMH. The ELISA plate was read (iMark™ Microplate Absorbance Reader, Bio-Rad, Santo Amaro, São Paulo, São Paulo, Brazil) at an absorbance of 450 nm. The average optical density (OD) was calculated for each calibrator, control, and sample. Thus, mean OD reading records for each calibrator along the y-axis versus AMH concentrations along the x-axis were determined using linear regression curve fitting. The samples with a concentration greater than the curve's upper limit were diluted and reanalyzed. The 1: 2,000 dilution was performed for D-60, 1: 5,000 for D-30 and D0, and 1: 200 for D+30) and D+60.

Variables were assessed for normality distribution using the Kolmogorov- Smirnov test, and the normality failed. AMH values were correlated with sperm characteristics using the PROC CORR Sperman of the SAS software (1992). The mean concentrations of the AMH were also compared in each evaluation period. The averages were compared using the Tukey test ($P < 0,05$). The correlation was considered as very weak (0-0.19), weak (0.2-0.39), moderate (0.40-0.59), as strong (0.6-0.79), and very strong (0.8-1) (Doria-Filho, 1999). For this proposal, the results were tabulated and presented as mean and standard deviation. The $P<0.05$ was considered for significative difference or correlation.

RESULTS

The means of the body weight and testicular measures were described in Table 1, and the sperm characteristics in Table 2.

Table 1. Mean ± standard deviation of body weight and testicular measures of the Nelore bulls during the peripubertal period

Period of the peripuberty	Body weight (kg)	Testicular volume (cm3)*	Scrotal circumference (cm)	Testicular height (cm)	Testicular width (cm)
D-60 (n= 8)	244.5±11.4	534.58±192.29	16.8±1.3	7.5±0.9	6.7±0.6
D-30 (n= 8)	250.8±12.5	806.98±293.20	17.6±1.4	8.3±1.0	7.8±0.7
D0 (n= 8)	259.4±13.6	1124.90±337.47	18.7±1.4	9.1±0.9	8.8±0.7
D+30 (n= 8)	271.3±15.8	1485.416±424.41	19.8±1.0	10.0±0.8	9.7±0.8
D+60 (n= 8)	283.5±18.0	1940.32±508.54	20.8±1.5	10.8±0.8	10.6±0.8

* This measure represents the volume of the two testicles according to Unanian *et al*. (2000).

Table 2. Mean ± standard deviation of the semen characteristics from the peripubertal Nelore bulls

Period of the peripuberty	Sperm Motility (%)	Sperm Vigor (1-5)	Total sperm Count (x10^6)	Sperm Count (x10^6/mL)	MD (%)	mD (%)
D-60 (n= 8)	--	--	--	--	--	--
D-30 (n= 8)	--	--	--	--	--	--
D0 (n= 8)	11.3±1.8	1.1±0.4	191.25±49.38	167.5±20.0	43.7±5.2	4.7±0.9
D+30 (n= 8)	51.9±0.9	2.6±1.06	776.25±487.73	270.0±14.2	20.4±4.7	8.1±134.4
D+60 (n= 8)	67.5±6.9	3.85±0.6	1556.25±332.94	398.8±24.6	7.9±1.6	13.8±3.1

MD: Major defects; mD: Minor defects; (--) No sample

There was a significative difference (P < 0.05) between D-60 and D+60 and between D0 and D+60 of the plasma concentrations of the AMH (Figure 1); however, no correlation was observed between semen characteristics and AMH concentrations (P > 0.05). Despite this, the testicular height (r = 0.69, P = 0.05) and width (r = 0.79, P = 0.01) at D0 were positive and strongly correlated with plasma concentrations of the AMH.

The bulls produced ejaculate at 10 months of age, with motility and sperm concentration >10% (11.25±2.31) and > $50x10^6$ spermatozoa (167.5±23.75), respectively, and weighting 259.38 ± 17.20kg.

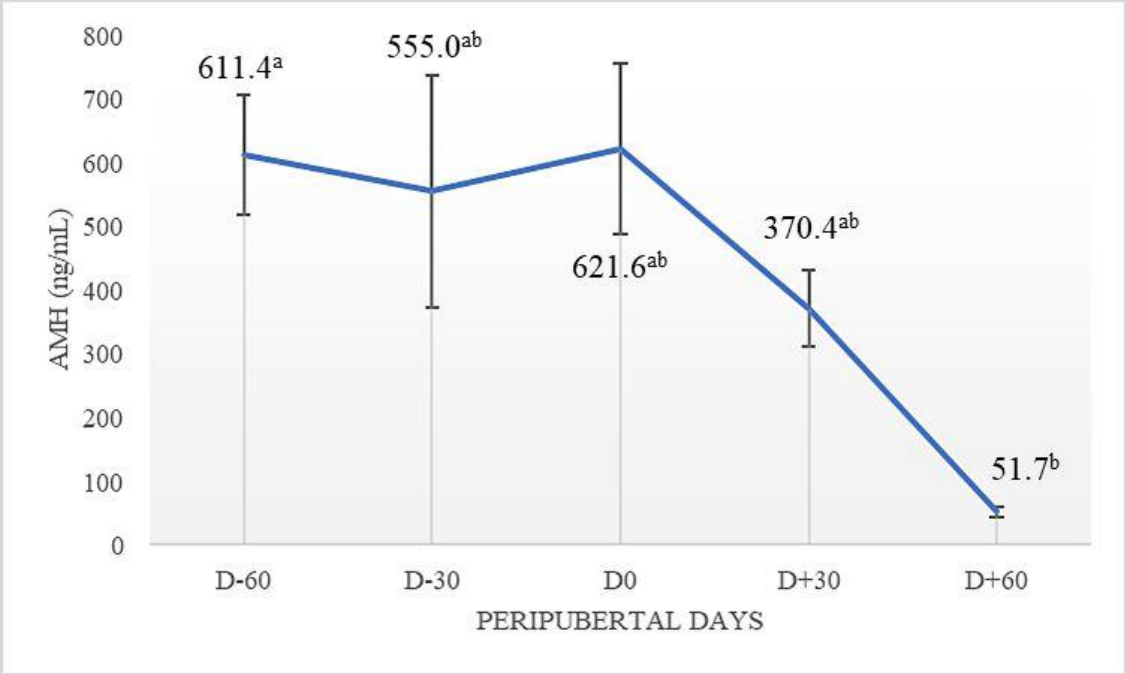

Figure 1. Peripubertal concentrations of the AMH of the Nelore bulls at peripubertal period.

DISCUSSION

For males, the main reproductive criterion used in genetic improvement programs has been scrotal circumference evaluated at different ages (Kluska *et al*., 2018). However, although the correlation between scrotal circumference and changes in seminal parameters is high for pubescent animals, it may not portray the reality of the testicular parenchyma (Bailey *et al*., 1998).

The semen evaluation was performed for 150 days, starting with the calf weaning at 8 months, approximately 60 days before puberty onset. The pubertal sample collection was established according to Castro *et al*. (1989), which mentioned in Nellore bulls the puberty between 12 and 14 months of age using the criteria cited by Wolf *et al*. (1965).

The calves classified as super-precocious, the puberty started at 10 months of age based on body weight, and scrotal circumference (Lunstra *et al*., 1978; Freneau *et al*., 2006) since the pubertal characteristics of the beef cattle (Angus breed) is in mean 273kg body weight, 28.6 cm scrotal circumference at 9.8 months of age (Vale Filho *et al*., 2001). Although the scrotal circumference was lower than in another study (Castro *et al*., 1991), we observed 31.2±2.4cm for characteristics, and the calves presented younger age and seminal characteristics like Nellore bulls at 24 months of age (Castro *et al*., 1991). The bulls produced ejaculate at 10 months of age, with motility and sperm concentration > 10% (11.25±2.31) and > 50x10 6 spermatozoa (167.5±23.75), respectively, and weighting 259.38±17.20kg, being characterized as early pubertal age (Wolf *et al.* 1965).

The early puberty can also be associated with food management (Romano *et al*., 2005), considering that calves were kept in paddocks with creep feeding, receiving 1% body weight of cattle ration. The management conditions like food and climate were reported to unfavorably influence Zebu calves to reach puberty, which can be corrected with food supplementation (Guimarães, 1993). Moreover, calves submitted to different nutritional food qualities reach puberty at different ages (Garcia *et al*., 1987). In animals fed with low food quality, puberty is later, and in the other growth phases, the nutritional factor did not influence the reproductive characteristics (Abdel-Raouf, 1960; Brito, 2014; Krieck *et al*., 2022). The feeding quality also modulates the central nervous system, including the endocrine system (Durlinger *et al*., 1999), which directly affects the reproductive functions, influencing both ages at puberty and seminal characteristics (Rekwot *et al*., 1988; Krieck *et al*., 2022).

Regarding AMH concentrations, the data collected at 9 months of age of the young bulls (D-30) differ from another study that found 16.14±1.47ng/mL in Nellore calves, weighting 271.95±35.35kg (Costa Filho *et al*., 2017). This difference may be attributed to the climate or genetics (Garcia *et al*., 1987) since our study was conducted in a tropical environment according to the Köppen classification.

AMH concentrations declined over time, reaching the lowest value at 60 days after puberty, which corroborated with other studies that mentioned the Sertoli cells produce high levels of AMH from fetal life during sexual differentiation and testicular development until puberty, a period that the hormone expression and concentration decrease (Josso *et al*., 1993; Rico *et al*., 2011; Rajak *et al.*, 2017). Despite this, we did not observe a correlation between the semen characteristics and plasma AMH concentrations, but testicular measures were correlated with the hormone concentration during puberty. Indeed, decreasing AMH production by Sertoli cells is related to increases spermatogenesis in the seminiferous tubules (Almeida *et al*., 2012), when the testicular cells reach maturity (Josso, 1992; Xu *et al*., 2019).

The reduction of the AMH production occurs at the same period that androgen receptor expression in testicular cells since AMH in high concentrations suppresses the P450c17 gene transcription, responsible for coding the steroid 17 alpha-hydroxylase/17,20 lyase enzymes that carry out steroidogenesis (Teixeira *et al*., 1999). Furthermore, there is a reduction in the AMH mRNA expression in the same proportion as the androgen receptors expression in Sertoli cells in horses (Almeida *et al*., 2012).

Interestingly Kitahara *et al*. (2016) found that AMH concentrations at puberty time were like our study. These researchers observed that at the puberty onset, the concentration of AMH remained at similar levels within the first two evaluations, and only after 30 days the levels dropped considerably.

Indeed, the decline of AMH plasma concentration at the peripubertal period takes place from 13 to 20 months due to its correlation to the Sertolli cells' maturation. Moreover, AMH reduction supports the proliferation of Leydig cells and testosterone production, which contributes to increased testicular maturation.

CONCLUSIONS

According to our results, AMH concentrations are high until puberty, decreasing significantly 30 days later, not being a reliable marker for puberty.

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