Theriogenology 89 (2017) 1–8

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Age-related changes in transcriptional abundance and circulating levels of anti-Mullerian hormone and Sertoli cell count in crossbred and Zebu bovine males

S.K. Rajak [a], A. Kumaresan [a,*], N.M. Attupuram [a], S. Chhillar [a], R.K. Baithalu [a], S. Nayak [a], L. Sreela [a], Raushan K. Singh [a], U.K. Tripathi [a], T.K. Mohanty [b], Savita Yadav [c]

[a] Theriogenology Lab, Animal Reproduction, Gynaecology and Obstetrics, National Dairy Research Institute, Karnal, Haryana, India
[b] Artificial Breeding Research Centre, National Dairy Research Institute, Karnal, Haryana, India
[c] Department of Biophysics, All India Institute of Medical Sciences, New Delhi, India

ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 27 September 2016
Accepted 3 October 2016

Keywords:
Anti-Mullerian hormone
Testis
Sertoli cell
Age
Crossbred
Zebu male

ABSTRACT

Age-related changes in peripheral anti-Mullerian hormone (AMH) concentrations and transcriptional abundance of AMH gene in testicular tissue were studied in crossbred (Holstein Friesian × Tharparkar) and Zebu (Tharparkar) males. In both the breeds, basal AMH concentrations were estimated using ELISA method in blood plasma obtained from six males each at 1, 6, 12, 18, and 24 months age. After blood collection at respective ages, all the males were castrated and expression and immunolocalization of AMH was performed in the testicular tissue. The concentration of AMH in blood plasma was found to be highest at 1 month of age in both crossbred and Zebu males, which subsequently decreased with advancing age. Significantly ($P < 0.05$) lower concentration of AMH was observed in crossbred as compared with Zebu males at 24 months of age. In line with peripheral AMH concentrations, the expression of AMH gene was also higher ($P < 0.05$) at 1 month of age, which thereafter declined significantly with advancement of age in crossbred males. Furthermore, the expression of AMH gene differed significantly between Zebu and crossbred males at all the age groups studied. Immunolocalization of AMH in testicular tissue also revealed a stronger expression at 1 month age, which gradually decreased till 24 months of age. The true Sertoli cell count was significantly higher in Zebu compared with crossbred males at all age groups studied except at 6 months age. The relationship between Sertoli cell count and circulating AMH concentrations was negative and significant ($r = -0.81$; $P = 0.004$). In conclusion, expression of AMH gene in testicular tissue and peripheral blood concentrations of AMH were higher in young compared with adults in both crossbred and Zebu males; however, the transcriptional abundance and circulating levels of AMH were higher in Zebu compared with crossbred males.

1. Introduction

Crossbreeding of low-producing indigenous cattle with high-producing European breeds like Holstein Friesian is followed in several developing countries to improve milk productivity. However, the males produced out of these crosses suffer from serious subfertility problems and the incidence of poor-quality ejaculates is very high in crossbred bulls [1–3]. Almost 50% of the ejaculates produced by the crossbred bulls did not fulfill the requirements of good-quality ejaculates [4]. The reason for production of poor-quality semen in crossbred bulls, even during the best

* Corresponding author. Tel.: +91 184 2259581; fax: +91 184 2250042.
E-mail addresses: ogkumaresan@gmail.com, A.Kumaresan@icar.gov.in (A. Kumaresan).

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http://dx.doi.org/10.1016/j.theriogenology.2016.10.003

breeding season, has not been identified yet. Increasing evidences indicate that the number of mature Sertoli cells in the testis, determines both testis size and daily sperm production [5], sperm quality [6], and has limited capacity to support the number of germ cells, which varies with species [7].

Sertoli cells play a critical role in spermatogenesis by providing hormonal signals, physical support, and necessary nutrients to spermatogenic cells. Immature Sertoli cells divide constantly, but this proliferative activity declines as puberty progresses. Recently, we have shown that the proportion of Sertoli cells in the testicular cytology had positive relationship with semen quality in bulls [6,8]. Further, proteomic analysis of Sertoli cells revealed differences between bulls/breeds with different fertility potential [9]. Less-mature Sertoli cells secrete abundant quantities of anti-Mullerian hormone (AMH) at the time of sexual differentiation of fetus until puberty [10,11]. AMH plays a major role during sexual differentiation and regression of Mullerian duct [12]. In human, it has been shown that serum AMH concentrations seem to constitute additional diagnostic parameters for male subfertility as they reflect Sertoli cell function [13]. Alterations in AMH secretion may lead to the development of abnormal conditions like anorchia and hypogonadism in males [14]. Secretion of AMH from Sertoli cell was reported to be higher till puberty and its expression severely decreased with the onset of spermatogenesis [10,12,15]. Further, it has been shown that the expression of AMH after puberty in male is an indication of failure in the maturation of Sertoli cells [16,17]. Although the concentrations of AMH in peripheral blood plasma have been studied in relation to ovarian follicular reserve and ovarian function in women [18,19] cow [20–22] and mare [23], expression of AMH gene and peripheral concentrations have not been studied in detail in bulls. Available information on AMH refers to the period of sexual differentiation of the fetus; however, information on AMH concentrations during prepubertal and pubertal period is scanty [24].

Because male subfertility is higher in crossbreds compared with Zebu cattle and AMH is being increasingly examined as a potential biomarker of reproductive efficiency in other species, including cattle [25], we hypothesized that there might be differences in peripheral concentrations of AMH and expression of AMH gene in the testicular tissue of crossbred and Zebu males. Thus, the aim of the present investigation was (1) to determine the age-related changes in the basal concentrations of AMH in peripheral blood samples in crossbred and Zebu males and (2) to study the transcriptional abundance of AMH gene in testicular tissue. Besides these, the age-related changes in Sertoli cell count were also studied in both the breeds.

2. Material and methods

2.1. Experimental animal and their management

The present experiment was carried out in crossbred (Holstein Frisian × Tharparkar crosses, exotic inheritance, 50%–75%) and Zebu (Tharparkar) males maintained at Livestock Research Center, National Dairy Research Institute, Karnal, India. Six males each at 1, 6, 12, 18, and 24 months of age were utilized for the study. All the animals in a particular age group were maintained at common management conditions as per the standards. The calves were fed with colostrum up to 5 days and then with whole milk, calf starter, and skim milk up to 3 months of age. Thereafter, the nutrient requirements of the animals were mostly met with *ad lib* green fodder and measured amount of concentrate ranging from 1.0 kg to 2.0 kg per animal for body maintenance. All the animals had free access to clean drinking water throughout the day. Vaccination, deworming, regular check-up for communicable diseases, and other herd-health programs were followed as per the farm schedule to protect the animals from diseases. Animals for the present experiment were approved by Institutional Animal Ethics Committee (IAEC).

2.2. Blood collection and AMH estimation

To estimate the basal AMH concentrations, three blood samples (at 1.5 hours intervals) were collected from each experimental animals on a single day at 1, 6, 12, 18, and 24 months age through jugular vein puncture using sodium heparin vacutainer tube (Vaccuette). Blood plasma was separated by centrifugation (3000 rpm for 10 minutes) at room temperature (25 °C) and stored at −20 °C till assay. Plasma AMH concentrations were estimated using bovine-specific AMH ELISA Kit (Cloud-Clone Corp., USA).

2.3. Expression of AMH gene in testicular tissue

The experimental animals were castrated at 1, 6, 12, 18, and 24 months age and expression and immunolocalization of AMH was performed in the testicular tissue. Before castration, the animals were sedated with xylazine hydrochloride (Xylaxin, Indian Immunologicals, India) at the dosage rate of 0.25 mL/50 kg body weight. Then, the testis was locally infiltrated with 5 to 8 mL 2% lignocaine (Cadila Healthcare Ltd., India) at the level of the spermatic cord. Incision was given at lower part of scrotum with the help of a BP blade (No. 23) with knife. Testis of each animal was exposed and the spermatic cord was ligated tightly using catgut (Size 3–0; Stericat Gutstrings (P) Ltd., India). After ligation, intact testicle was incised, removed, and placed in individual sterile containers containing normal saline with penicillin streptomycin. All the bulls were given due post-operative care as per the standard veterinary protocol. Immediately after castration, about 100 mg of testicular parenchyma was collected and placed in RNA stabilization reagent (RNAlater, Qiagen, Ambion, Austin, USA) and stored at −80 °C till further use.

2.4. RNA isolation and complementary DNA synthesis

The isolation of RNA from testicular tissues was performed using RNeasy minikit(Qiagen, Ambion, Austin, USA). The quality and quantity of RNA were checked in agarose gel electrophoresis (1.5%) and NanoQuant Infinite M200 PRO (TECAN). All samples had an A 260/280 absorbance ratio between 1.85 and 2.0. The concentrations of RNA from all the samples were normalized to 500 ng/uL for complementary DNA (cDNA) synthesis. Preparation of