Original Research

Age-Specific Peripheral Anti-Mullerian Hormone (AMH) Concentration: A Candidate Endocrine Marker for Fertility Assessment in Cattle

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Abstract

Anti-mullerian hormone (AMH) emerges as the most reliable endocrine marker in assessing the fertility potential over the ages. The age-specific reference range for peripheral AMH concentrations in cattle is lacking. The present study thus aimed (i) to address the question whether ovarian secretion of AMH would be affected by gonadotrophic status after gonadotrophin releasing hormone (GnRH) challenge in cattle and (ii) to establish age-specific plasma AMH concentrations in Holstein Friesian crossbred cattle (n= 151) using an enzyme immune-assay technique. Data on hormonal concentrations over time in GnRH-treated animals were analyzed by nonparametric one-way repeated measure ANOVA. GnRH challenge could not bring any change (P>0.05) in plasma AMH concentrations, while plasma FSH and LH concentrations changed significantly (P<0.01) over time after GnRH administration in cattle. Data on age specific hormonal concentrations in cattle were analyzed by nonparametric one-way ANOVA, i.e. Kruskal-Wallis test to find the significant change of plasma AMH concentrations over time, if any, in cattle. Plasma AMH concentrations changed significantly (P<0.05) between the mean rank of the related groups over the time (age) in cattle. A cubic model was found to be the best fitted model for explaining the change of plasma AMH level with age. The present results open the possibility of using the peripheral AMH level as a candidate endocrine marker for the assessment of reproductive status.

Key words: Anti- Mullerian Hormone, AMH in Cattle, Endocrine Marker, GnRH Challenge, Ovarian Aging

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Introduction

Fertility potential is of immense practical significance in livestock farming business. The common concept of female reproductive aging is the loss of quantity and quality of oocyte/ follicle pool in the ovaries. Ovaries suffer more serious effect of aging than any other tissues of the female body (Amanvermez and Tosun, 2016). During the aging process, both the number and quality of the oocytes in the ovaries diminish and attain to a point beyond that, cyclic endocrinological activities stop, entering the menopause that indicates the absolute end of reproductive life in women. Though similar kind of episode happens in farm animal species; however, menopause like stage has not been coined in farm animal species. The classical view of a finite primordial follicle pool in the ovaries called ovarian reserve provides better understand of ovarian aging (Broekmans *et al*., 2009). The ovarian reserve declines gradually with increasing chronological age. At the birth of a new born female calf, the number of healthy follicles and oocytes in ovaries varies from 10,000 to 3,50,000 (Erickson, 1966) and this number reaches between 1,920 and 40,960 at 12-month-old heifers and the rest of the follicles are lost by apoptosis (Ireland *et al.*, 2008). As oocyte quantity rapidly declines with increasing age, average conception rate following artificial insemination (AI) of Holstein heifers declines from a peak of 56% in 15- 16 months of age to 42% at 26- 27 months of age (Kuhn *et al*., 2006). Thus, the age- related decline of ovarian follicular reserve is a major determinant of reproductive aging.

Early follicular phase serum levels of FSH, inhibin B, and estradiol are measured to assess an individual's ovarian reserve in farm animals. However, these endocrine markers are not independent of each other, as they represent the classical hypothalamus-pituitary-gonadal feedback loop. Hence, the routine use of these hormones is not recommended (Jirg, 2011). There is currently no reliable genetic marker of ovarian reserve that can be used as a routine test (Amanvermez and Tosun, 2016).

Presently, anti- mullerian hormone (AMH) appears to be the best endocrine marker in assessing the age-related decline of the ovarian reserve (Van Rooij *et al*., 2005) and predicting the ovarian response of induced human patients during *In vitro* fertilization (Grynnerup *et al*., 2012). There is a promising use of AMH as an endocrine marker for ovarian follicular reserve (Van Rooij *et al*., 2002), ovarian aging (de Vet *et al*., 2002) and ovarian responsiveness in assisted reproductive technology (Elgindy *et al*., 2007) in the field of human reproductive biology. AMH, a dimeric glycoprotein of 140 kDa, is a member of the transforming growth factor beta (TGF-β) family of growth that expressed by granulosa cells of pre-antral and early antral follicles of the ovary during the female reproductive life span and is proportional to the follicle population and is not influenced by gonadotrophic status in human (Fanchin *et al*., 2003; La Marca *et al*., 2005). However, it is not known whether peripheral AMH concentration responses in farm animals after gonadotrophin releasing hormone (GnRH) stimulation. Unlike women, the reference value for peripheral AMH concentration for defining the age-related ovarian senescence and/ or ovarian functional status in cattle is lacking. Therefore, the objectives were (i) to investigate the responsiveness of ovarian AMH to

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GnRH stimulation and (ii) to determine the age-specific reference range for peripheral AMH concentrations in cattle.

Material and Methods

The experimental protocol and animal care were met in accordance with the National guidelines for care and use of Agricultural Animals in Agricultural Research and Teaching as approved by the Ethical Committee for Animal Experiments (ECAE) of ICAR Research Complex for NEH Region, Barapani, Meghalaya, India.

GnRH Challenge Test

An experiment was designed to reveal the responsiveness of the ovary to GnRH challenge in cattle at Livestock Farm of Indian Council of Agricultural Research (ICAR) Complex for North Eastern Hill (NEH) region, Lembucherra, West Tripura, India located at 22°56′N latitude and 90°09′E longitude. Three non-pregnant and non-cyclic Holstein Friesian crossbred cattle with mean (± SEM) age of 8.40 ± 0.36 years were selected for the study. The animals selected were free from any anatomical, physiological, or infectious disorders. Following local anesthesia (Xylocaine-2%, Astra Zeneca, India), the animals were fitted with an indwelling catheter in a jugular vein. Before insertion, catheters were flushed with 100 IU of sodium heparin to prevent blood clot formation. The animals were administered intravenously with GnRH @ 0.25 µg/ kg body weight (Receptal®, M/s. Intervet, India) as this dosage of GnRH previously elicited a large increase in plasma FSH and LH in both cows and goats (Fajersson *et al*., 1999; Haldar *et al*., 2013). The serial blood samples were collected in heparinised tubes on -60, -30, 0 min (0 being the time of GnRH administration) and then every 30 min interval for 9 h post GnRH administration. After removal of the catheter, the animals were treated with antibiotic, multivitamins and calcium for 3 days considering the health and welfare of the animals. The blood samples were centrifuged at 2500×*g* for 10min at 4^0C and the plasma was separated and stored at -20^0C until hormone assay.

Farm Animals and Blood Sampling

A total of 151 Holstein Friesian crossbred cattle covering different age groups were selected during a period of 8 months from two Livestock Farms located at ICAR Research Complex for NEH region, Lembucherra, West Tripura and National Dairy Research Institute (NDRI), Karnal, Haryana. Based on the farm record, 57 animals of ICAR Research Complex for NEH region's Livestock Farm, Lembucherra, West Tripura and 94 animals of NDRI Farm were selected randomly and grouped under different age groups started from three months old and then one year old, two years old and up to fifteen years of age. The animals were housed and maintained in a sheltered paddock under natural daylight and environmental conditions. They were fed according to the standard feeding regimen on the farm with an access to green fodders and

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commercially available concentrate feed. Clean drinking water was made available *ad libitum.* Deworming and vaccination were done as per standard schedule. The animals were checked clinically and confirmed that they were free from any anatomical, physiological or infectious disorders. The adult animals were either cyclic or pregnant. A single blood sample was collected from each selected animal before feeding between 08:30h and 09.30h by jugular venepuncture into heparinised polypropylene tubes (20 IU heparin/ml of blood) taking due care. Plasma samples were collected after centrifugation at 2500×*g* for 10 min at 4°C and stored at -20°C until hormone assay.

Hormone Assay

Plasma LH concentration in GnRH treated cattle were estimated using a double antibody and biotin–streptavidin peroxidase amplification system in a competitive-binding enzyme immunoassay (EIA) as previously validated (Prakash *et al*., 2002). Eighty microliter of undiluted plasma sample in duplicate was run in 96-well microtiter plate for the assay. An automated micro-titer plate washer (Model: W-2002, Electronic Corporation of India Ltd., India) was used to wash the microtiter plate. Absorbance by the yellow colour obtained after the enzyme–substrate reaction was measured at 450 nm with the aid of an 8-channel automatic 96 well microtiter plate reader (Model:MS5605A, Electronic Corporation of India Ltd., India). A representative standard curve for EIA to determine plasma LH in cattle is presented in Fig. 1. The intra- and inter-assay coefficients of variation (CVs) were 7.9% and 10.3%, respectively. The sensitivity of the EIA assay for LH was 0.3 ng/ml.

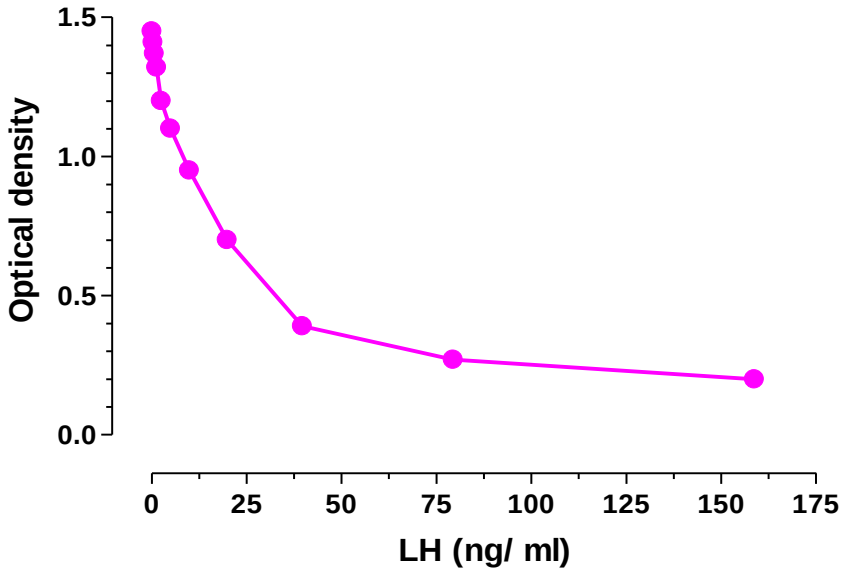

Fig. 1: A representative standard curve for the enzyme immunoassay to determine plasma LH in cattle

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Plasma FSH concentration in GnRH treated cattle was quantified in 96-well microtiter plate using horseradish peroxidase (HRP) enzyme conjugate based on competitive binding method on a solid-phase EIA of commercially available kits for bovine (M/s. Endocrine Technologies, Inc., USA). Fifty microliter of undiluted plasma sample in duplicate was used to quantify plasma FSH concentration by an EIA using an 8-channel automatic 96 well microtiter plate reader (Model:MS5605A, Electronic Corporation of India Ltd., India). A representative standard curve for EIA to determine plasma FSH in cattle is presented in Fig. 2. The sensitivity of the assay for FSH was 0.5 ng/ml. The intra- and inter-assay coefficients of variation (CVs) were 8.8% and 11.6%, respectively.

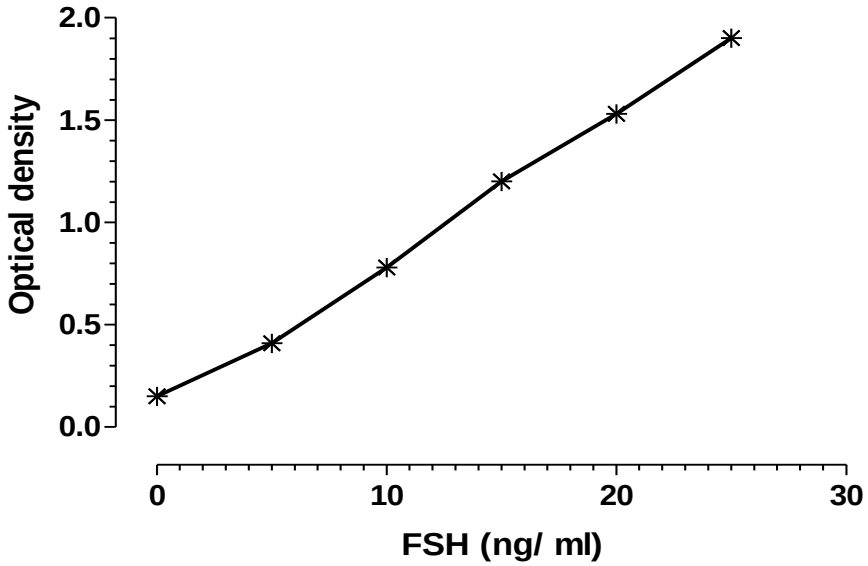

Fig. 2: A representative standard curve for the enzyme immunoassay to determine plasma FSH in cattle.

Plasma concentration of AMH was determined using a solid-phase EIA of commercially available bovine AMH (bAMH) kit (M/s. Novateinbio Biosciences, USA) in 96-well microtiter plate based on competitive binding method. The concentrations of AMH were determined in 50 µl samples of undiluted plasma in duplicate. An 8-channel automatic 96 well microtiter plate reader (Model:MS5605A, Electronic Corporation of India Ltd., India) was used to read absorbance of the yellow colour obtained after the enzyme–substrate reaction at 450 nm. A representative standard curve for the enzyme immunoassay to determine plasma AMH in cattle is presented in Fig. 3. The lowest plasma AMH detection level was 0.25 ng/ ml. The intra- and inter-assay coefficients of variation (CVs) were 8.9% and 12.3%, respectively.

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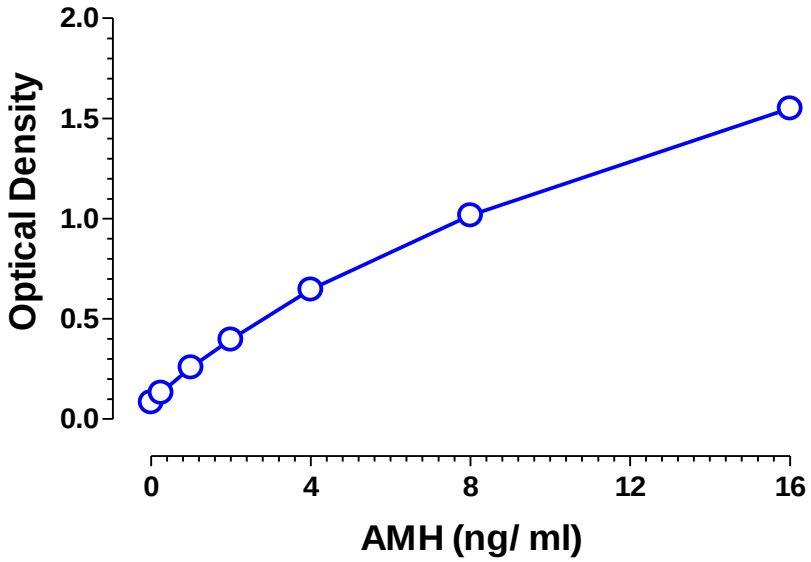

Fig. 3 A representative standard curve for the enzyme immunoassay to determine plasma AMH in cattle.

Statistical Analysis

All statistical analyses were performed using SAS 9.3 Statistical Software Package, 2012 (SAS 9.3, 2012). Data are presented in the text as the mean ± the standard error of the mean (SEM). Data on hormonal concentrations (plasma FSH, LH and AMH) over time in GnRH-treated cattle were analyzed by nonparametric one-way repeated measures ANOVA, i.e. Friedman test to find the significant effect of GnRH administration on FSH, LH and AMH over time in cattle. Plasma AMH data of cattle at different ages were analyzed by PROC LOGISTIC (SAS 9.3, 2012) to fit Logit model for finding out any location effect on plasma AMH data, if any. The location effect was not statistically significant ($P>0.05$). Thus, there was no evidence of location effect on plasma AMH concentrations in cattle. Hence, data on plasma AMH concentrations over time in cattle recorded on 151 cattle in two locations, viz. ICAR Research Complex for NEH region, Lembucherra, West Tripura and NDRI, Karnal, Haryana were pooled together and subjected to nonparametric one-way ANOVA i.e. Kruskal-Wallis test to find the significant change of plasma AMH concentrations over time, if any, in cattle.

Plasma AMH concentrations over time for cattle were fitted to find the best fitted model for defining the relationship between plasma AMH level and age. Among the different models, the following cubic model was found to be the best fitted model with respect to the R-square criteria.

$$y = b_0 + b_1x + b_2x^2 + b_3x^3$$

where y= dependent variable, x= independent variable and b_0, b_1, b_2, b_3 are the coefficients of the model.

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Results and Discussion

Plasma FSH, LH and AMH Profiles in Response to GnRH Challenge Test

The mean (± SEM) plasma FSH, LH and AMH profiles in cattle in response to GnRH challenge test are presented in Fig. 4. Nonparametric one-way repeated measure, i.e. Friedman test revealed that plasma FSH and LH concentrations changed significantly (P<0.01) over time after GnRH administration in cattle. Following GnRH administration, there was an increase in plasma FSH concentration just after 30 min. Thereafter, plasma FSH concentrations started increasing and reached at the peak value between 210- and 240-min post GnRH administration and again, it declined gradually till 480 min post GnRH administration. Plasma LH concentration started increasing within 30 min and reached at the peak value at 120 min post GnRH administration and then it declined gradually till 480 min post GnRH administration. There was no change of plasma AMH concentrations (P = 0.896) after GnRH challenge.

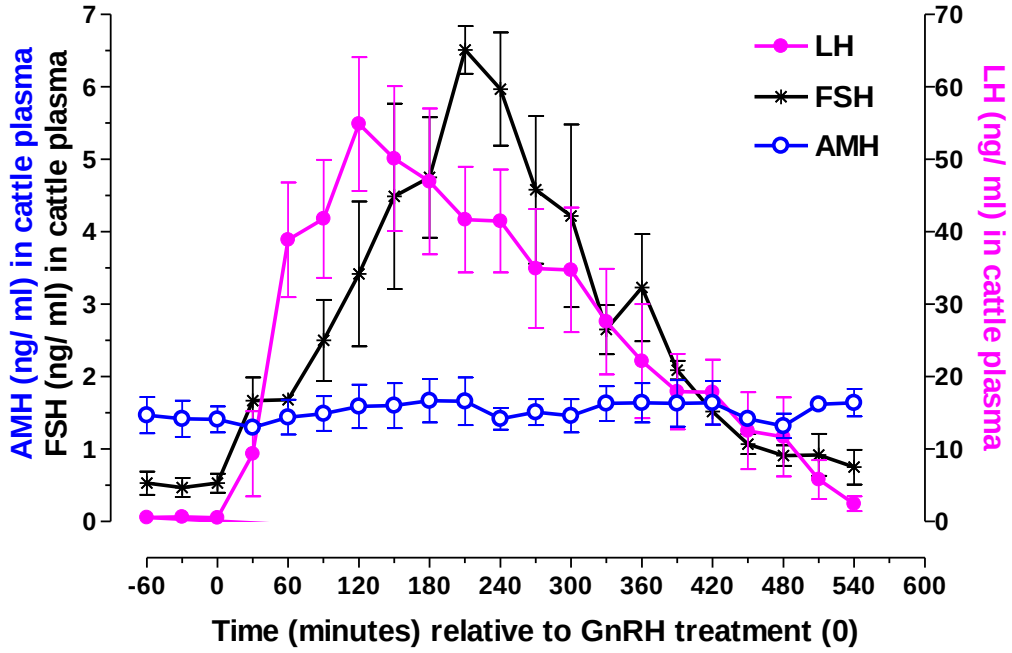

Fig. 4: Plasma AMH, FSH and LH profiles in cattle (n= 3) after GnRH challenge. Time 0 represents the time of GnRH administration intravenously @ 0.25 µg/ kg body weight.

In the present study, the hypothesis was tested whether GnRH challenge induced pituitary secretions of FSH and LH could influence AMH secretion from ovary in Holstein Friesian crossbred cattle. The significant change (P<0.01) over time in pituitary secretions of FSH and LH after GnRH administration in cattle is quite similar to that of previous experiment on cattle (Fajersson *et al*., 1999). GnRH is a key regulator of reproductive functions, which triggers the synthesis and release of FSH and LH from the pituitary gland (Schneider *et al*., 2006). The present study showed that the plasma AMH level was

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independent of pituitary FSH and/ or LH action. To the best of the current knowledge, no effect of GnRH on the ovary to produce AMH in cattle is the first report. Earlier report indicated treatment of IVF patients with a single, high dose of GnRH agonist, resulting in a rise of endogenous FSH and LH, did not affect serum AMH levels (Van Rooij *et al*., 2002). Administration of exogenous hormones during estrus synchronization could not affect plasma AMH concentrations in cattle (Pfeiffer *et al*., 2014). This could be due to the fact that AMH was not involved in feedback mechanisms of the hypothalamus-pituitary-gonadal axis (Visser *et al*., 2012). AMH concentrations remained constant during pregnancy (La Marca *et al*., 2005) and during ovarian cycle (Rico *et al*., 2011), explaining why a single AMH measurement has been usually sufficient. Hence, a single AMH measurement in the plasma of animals at any age or physiological stage could be useful for the assessment of reproductive status in cattle.

Plasma AMH Profiles in Farm Animals

In the present study, the parameter estimates of the best fitted cubic model are presented in Table 1. As shown in Fig. 5, a cubic model was found to be the best fitted model that delineated the decline of plasma AMH with age.

Table 1: Best fitted model for plasma AMH concentrations in cattle over time in age

Animal Species	Best Fitted Model	R-square	Fitted Model
Cattle	Cubic	0.939	$y = -0.462 + 1.220x - 0.140x^2 + 0.004x^3$

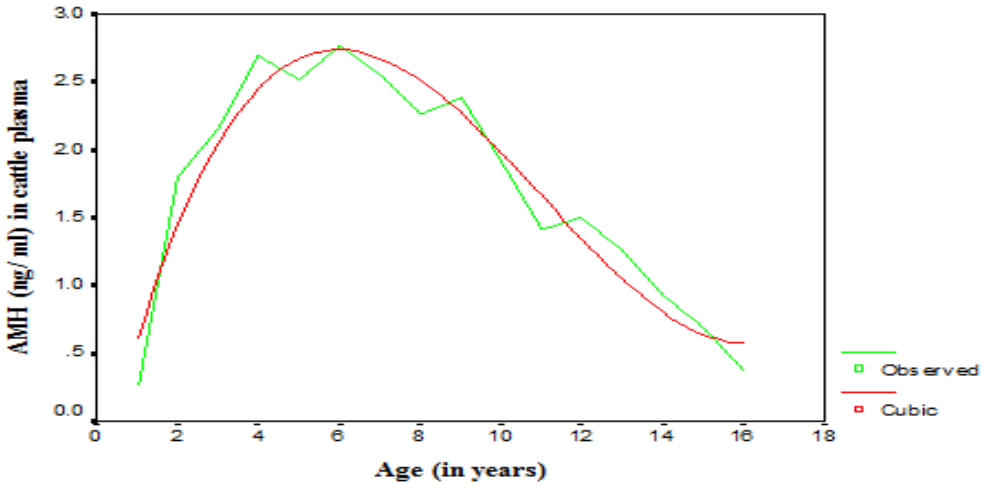

Fig. 5: The best fitted cubic model curve is showing the trend of plasma AMH concentrations (ng/ ml) in cattle during a period from 3 months to 15 years of ages.

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Plasma AMH profiles covering different ages in cattle are presented in Fig. 6. Nonparametric one-way ANOVA i.e. Kruskal-Wallis test revealed that AMH concentrations changed significantly ($P<0.05$) between the mean rank of the related groups over time. Mean (± SEM) plasma AMH concentration at the age of 3 months was just detectable (0.26 ± 0.03 ng/ ml) and thereafter it increased till 3 years of age and then remained same (≥ 2 ng/ ml) with slight fluctuations till 8 years of age and again it started a progressive decline till 15 years of age (0.37 ± 0.10 ng/ ml).

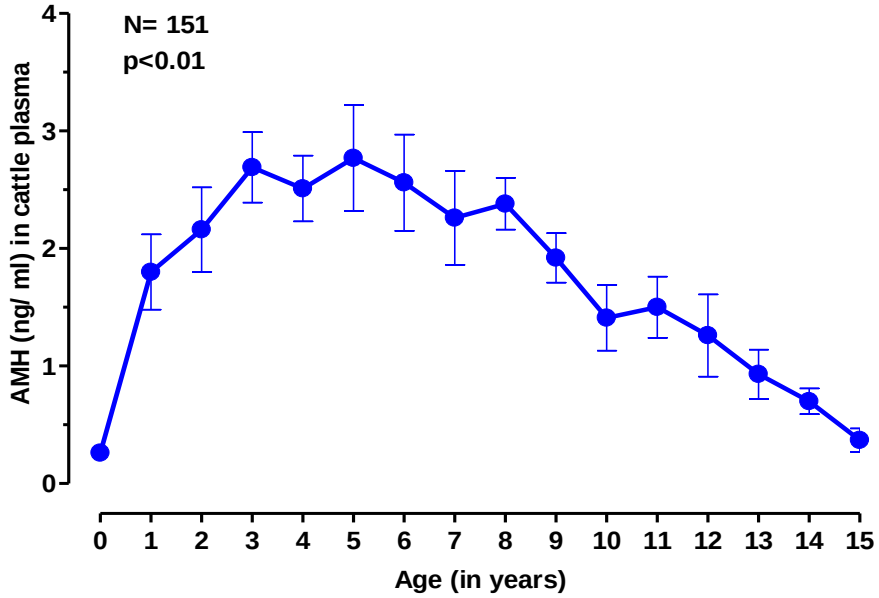

Fig. 6: Plasma AMH concentrations (ng/ ml) in cattle (n= 151) from 3 months to 15 years of ages.

The vast majority of studies on AMH has been documented in rodents and human. AMH appeared to be the best endocrine marker in assessing the age-related decline of the ovarian follicular reserve in mice (Kevenaar *et al*., 2006) and in humans (Christiansen *et al*., 2016). A recent study established the age-specific normal reference ranges for serum AMH levels in a large population-based sample of healthy Chinese women (Du *et al*., 2016). The present study determined the age-specific plasma AMH concentrations in Holstein Friesian crossbred cattle for the first time. Circulating AMH concentration in young adult dairy heifers has been found to be a simple reliable biomarker to predict productive herd life in dairy cattle (Jimenez-Krassel *et al*., 2015) and select good embryo donors in embryo production programs of buffaloes (Liang *et al*., 2016), sheep (Lahoz *et al*., 2014), goats (Monniaux *et al*., 2011) and determine litter size in bitches (Hollinshead *et al*., 2017). Plasma AMH concentration has been reported to have a high degree of correlation with ovarian antral follicle count in cattle (Batista *et al*., 2014, 2016) and buffaloes (Baldrighi *et al*., 2014). Because AMH levels are strongly correlated with the ovarian follicular reserve, plasma AMH levels may be a candidate endocrine marker for assessing individual reproductive status in cattle. The

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present study provides a first-hand information on age-specific peripheral AMH levels in Holstein Friesian crossbred cattle.

Conclusion

The current study confirms that: i) GnRH stimulated pituitary FSH and LH release does not have any influence on ovary to produce AMH in cattle; and ii) a cubic model is the best fitted model that explains the change of plasma AMH concentrations with age in cattle. A single AMH measurement in the plasma of animals may open the possibility of using peripheral AMH concentration as a candidate endocrine marker. Hence, the current study would allow the farmers to test peripheral AMH concentration in cattle for knowing the expected reproductive potential in their animals.

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