Journal of Animal Research: v.10 n.5, p. 725-732. October 2020

DOI: 10.30954/2277-940X.05.2020.8

Age-Specific Peripheral Anti-Müllerian Hormone Concentration in Buffaloes

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Received: 27 Aug., 2020 **Revised:** 19 Sept., 2020 **Accepted:** 15 Oct., 2020

ABSTRACT

Anti- Mullerian hormone (AMH) is one of the important endocrine markers for the assessment of the age-related decline of fertility potential in animals. No baseline data is available on peripheral AMH concentrations at different ages in buffaloes (*Bubalus bubalis*). The present study thus aimed (i) to examine the responsiveness of the ovary to gonadotrophin releasing hormone (GnRH) challenge to release plasma AMH into circulation to address the question whether ovarian secretion of AMH would be affected by gonadotrophic status and (ii) to determine plasma AMH concentrations at different ages in buffaloes (n= 154) using an enzyme immune-assay. Data on hormonal concentrations in GnRH-treated buffaloes and in different ages of buffaloes were analyzed by non-parametric one-way repeated measure ANOVA and one-way ANOVA, respectively. No change ($p>0.05$) of plasma AMH concentrations after GnRH challenge in experimental buffaloes was recorded. Plasma AMH concentration was significantly lower ($p<0.05$) at 3 months of age (0.56 ± 0.29 ng/ ml) and thereafter it started increasing and reached at a level of 3.07 ± 0.44 ng/ ml at 3 years of age and then remained same (≥2 ng/ ml) up to 11 years of age and again declined progressively and reached at the level of 0.52 ± 0.12 ng/ ml at 15 years of age. A cubic model was the best fitted model to elucidate the change of plasma AMH level with age. Hence, the present study suggested that peripheral AMH concentration could be a candidate endocrine marker for the assessment of reproductive status in buffaloes.

HIGHLIGHTS

- Age-specific peripheral anti-müllerian hormone concentration for the assessment of reproductive status in buffaloes.
- Plasma AMH concentration is not influenced by hypothalamo-pituitary-gonadal axis or the gonadotropic status.
- A cubic model fitted best the decline of plasma AMH concentration with age in buffaloes.

Keywords: Anti- Mullerian hormone, GnRH challenge, age- related change of AMH, buffaloes

The evaluation of fertility potential in economic livestock animals is of great practical importance in animal husbandry. The common concept of female reproductive aging is the loss of quantity and quality of oocyte/follicle pool and thus the decline of reproductive potential. Ovaries undergo much 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 decrease and reach to a point beyond that, no more viable offspring may be produced and the associated cyclic endocrinological activities cease, entering the menopause in females. In women, menopause indicates the absolute end of reproductive life. However,

How to cite this article: Haldar, A., De, S., Gautam, D., Pal, P. and Brar, P.S. (2020). Age-specific peripheral anti-müllerian hormone concentration in buffaloes. *J. Anim. Res.,* **10**(5): 725-732.

Source of Support: None; **Conflict of Interest:** None

menopause like stage has not been coined in farm animal species. There is a classical view of a finite primordial follicle pool in the ovaries called ovarian reserve to better understand ovarian aging (Broekmans *et al*., 2009). This ovarian reserve declines progressively with increasing chronological age within expected ranges. At the birth of a newborn 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, 2006). The age-related decline of ovarian follicular reserve is a major determinant of reproductive aging. However, the quantity of follicles and oocytes is highly variable throughout the reproductive life spans of females (Lahoz *et al*., 2014). Hence, it makes a challenge to evaluate an individual female's reproductive potential for economic reason in livestock farming.

To assess an individual's ovarian reserve, early follicular phase serum levels of FSH, inhibin B, and estradiol are measured in farm animals. Age-related decrease in the number of oocytes leads to a decrease in estradiol and inhibin B levels, as a result of which FSH levels rise. However, these markers constitute the classical hypothalamus-pituitary-gonadal feedback loop and therefore, are not independent of each other. Hence, the routine use of these hormones is not recommended (Jirge, 2011). There is currently no reliable genetic marker of ovarian reserve that can be used as a routine test (Amanvermez and Tosun, 2016).

Currently, anti-mullerian hormone (AMH) appears to be the best endocrine marker in assessing the age-related decline of the ovarian pool (van Rooij *et al*., 2005) and predicting the ovarian response of induced human patients, including poor and hyper-responses of *in vitro* fertilization (Grynnerup *et al*., 2012). AMH, a dimeric glycoprotein of 140 kDa, is a member of the transforming growth factor beta (TGF-b) family (Visser *et al*., 2012) and expressed by granulosa cells of pre-antral and early antral follicles of the ovary 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 cannot be ruled out the possibility that a variation in peripheral AMH profiles may exist in farm animals after gonadotrophin releasing hormone (GnRH) stimulation. Thus, the measurement of serum AMH levels has been added to the panel of markers for ovarian aging (de Vet *et al*., 2002; Fanchin *et al*., 2003), ovarian follicular reserve (van Rooij *et al*., 2002) and ovarian responsiveness in assisted reproductive technology (Elgindy *et al*., 2007) in the field of human reproductive biology. Unlike human beings, the reference to plasma AMH concentrations to understand the ovarian functional status or reproductive potential at any point of age in farm animals is wanting.

Of particular interest was to study peripheral AMH profiles in Murrah buffaloes in the present study. India ranks first in the world to have the highest number of buffaloes (108.7 million) that are representing 58.0 % of the world's buffalo population and contributing about 55% of the total milk production in India (Annual Report, 2015- 16). Indian Murrah buffaloes (*Bubalus bubalis*) are the best milch buffalo breed in the world. Therefore, our objectives were (i) to investigate the responsiveness of ovarian AMH to GnRH stimulation and (ii) to determine the age-specific reference for peripheral AMH levels in Murrah buffaloes.

MATERIALS AND METHODS

GnRH challenge test

An experiment was designed to elucidate the responsiveness of the ovary to GnRH challenge in buffaloes at a 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 Murrah she buffaloes selected for the study were non-pregnant (empty) and non-cyclic with mean (± SEM) age of 8.57 ± 0.15 years. She buffaloes selected were free from any anatomical, physiological, or infectious disorders. The animals were fitted with an indwelling catheter in a jugular vein after local anesthesia (Xylocaine-2%, Astra Zeneca, India). Before insertion, catheters were flushed with 100 IU of sodium heparin to prevent blood clot formation. The animals were administered intravenously (i.v.) with GnRH @ 0.25 mg/ kg body weight (Receptal®, M/s. Intervet, India) as this dosage of GnRH previously elicited a large increase in plasma FSH and LH in cows

(Fajersson *et al*., 1999). 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 10 min at 4°C and the plasma was separated and stored at -20°C until hormone assay. 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.

Farm animals and blood sampling

A total of 154 Murrah buffaloes covering different age groups were selected during a period of 11 months from two livestock farms located at National Dairy Research Institute (NDRI), Karnal, Haryana and Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. Based on the farm record, 98 animals of NDRI Farm and 56 animals of Guru Angad Dev Veterinary and Animal Sciences University's 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 commercially available concentrate feed. Clean drinking water was made available *ad libitum*. Deworming and vaccination were done as per standard schedule.

Based on the farm record, the animals 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 year of age. The animals were checked clinically and confirmed that they were free from any anatomical, physiological or infectious disorders. The adult animals were cyclic. A single blood sample was collected from each selected animal before feeding between 08:30 and 9.30 h 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 buffaloes 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. The intra- and inter-assay coefficients of variation (CVs) were 7.8% and 10.5%, respectively. The sensitivity of the EIA assay for LH was 0.3 ng/ml.

Plasma FSH concentration in GnRH treated buffaloes was quantified in 96-well microtiter plate using horseradish peroxidase (HRP) enzyme congugate 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. The specificity of bovine FSH antigen and endogenous FSH in buffalo plasma for bovine FSH antibody was assessed by determining parallelism between bovine FSH standards and endogenous buffalo plasma FSH. Plasma samples containing high concentrations of endogenous FSH were collected from three GnRH treated buffaloes and pooled. The pooled plasma sample was serially diluted with assay buffer to obtain plasma volumes of 1: 0, 1: 1, 1: 2, 1: 4, 1: 8 and 1: 16 and run in an assay along with bovine FSH standards (ranging from 0.5 to 25 ng/ ml prepared in the assay buffer). The optical density obtained from increasing dilutions of buffalo plasma samples was parallel to that obtained with the bovine FSH standards as shown in Fig. 1. Thus, the test confirmed a considerable homology between buffalo and bovine FSH used in the assay. Hence, the assay provided an actual FSH determination in buffalo plasma. The sensitivity of the assay for FSH was 0.5 ng/ml. The intra- and inter-assay coefficients of variation (CVs) were 8.6% and 11.7%, respectively.

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. The lowest plasma AMH detection level was 0.25 ng/ ml. The intra- and inter-assay coefficients of variation (CVs) were 9.6% and 13.1%, respectively.

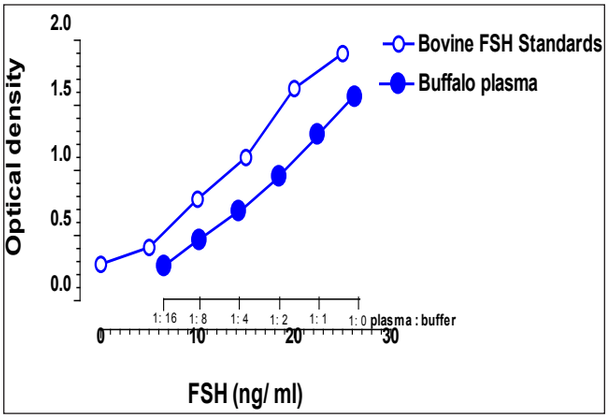

Fig. 1: The curve (˜) obtained from the pooled buffalo plasma sample serially diluted in buffer (1: 0, 1: 1, 1: 2, 1: 4, 1: 8 and 1: 16 plasma: buffer) was parallel to the curve (O) obtained with the bovine FSH standards (ranging from 0.5 to 25 ng/ ml) in the buffer. Optical density was measured at 450 nm.

Parallelism test between bAMH and buffalo plasma AMH

In order to validate the assay in buffalo plasma, the specificity of bovine AMH antigen and endogenous AMH in buffalo plasma for bovine AMH antibody was assessed by conducting parallelism test between bovine AMH standards and endogenous buffalo plasma AMH. Buffalo plasma samples containing high concentrations of endogenous AMH were serially diluted with assay buffer to obtain plasma volumes of 1: 0, 1: 1, 1: 2, 1: 4, 1: 8, 1: 16 and 1: 32 and run in an assay along with bovine AMH standards (ranging from 0.25 to 16 ng/ ml prepared in the assay buffer).

STATISTICAL ANALYSIS

All statistical analyses were performed using SAS 9.3 Statistical Software Package, 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 buffaloes were analyzed by non-parametric 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 buffaloes. Plasma AMH Data of buffaloes 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 buffaloes. Hence, data on plasma AMH concentrations over time in buffaloes recorded on 154 buffaloes in two locations, viz. 98 animals of NDRI Farm and 56 animals of Guru Angad Dev Veterinary and Animal Sciences University's Farm 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 buffaloes.

The AMH concentrations over time for buffaloes 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.

$$y = b_0 + b_1 x + b_2 x^2 + b_3 x^3$$

where,

y = dependent variable,

x = independent variable, and

b_0, b_1, b_2, b_3 are the coefficients of the model.

RESULTS AND DISCUSSION

bAMH parallelism with buffalo plasma AMH

The homology between bAMH standards and endogenous AMH in buffalo plasma was assessed by conducting parallelism test. The optical density obtained from increasing dilutions of buffalo plasma samples was parallel to that obtained with the bovine AMH standards as shown in Fig. 2. Thus, the test confirmed a considerable homology between buffalo and bovine AMH used in the assay. Hence, the assay provided an actual AMH determination in buffalo plasma.

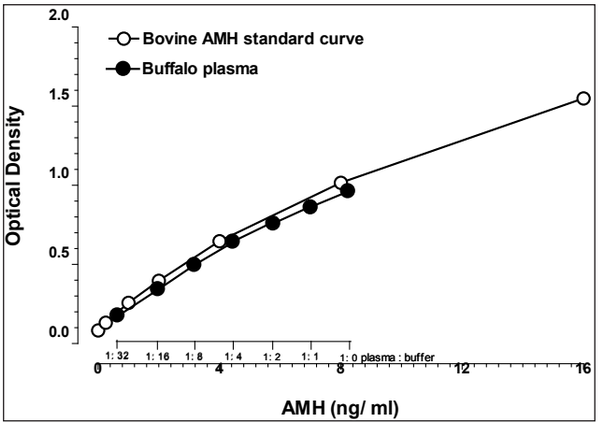

Fig. 2: Parallelism of curves (O) for bovine AMH standards (ranging from 0.5 to 16 ng/ ml) with serially diluted volumes (1:0, 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 for plasma: buffer) of buffalo plasma (˜). Optical density was measured at 450 nm

Plasma FSH, LH and AMH profiles in response to GnRH challenge test

The mean (± SEM) plasma FSH, LH and AMH profiles in buffaloes in response to GnRH challenge test are presented in Fig. 3.

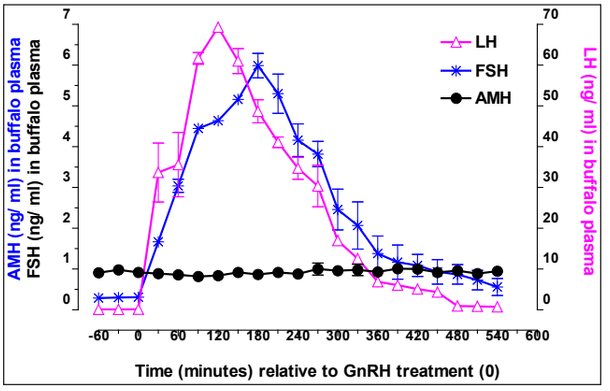

Fig. 3: Plasma AMH, FSH and LH profiles in buffaloes (n= 3) after GnRH challenge. Time 0 represents the time of GnRH administration intravenously @ 0.25 mg/ kg body weight

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 buffaloes. Following GnRH administration, there was an increase in plasma FSH concentration just after 30 min in experimental buffaloes. Thereafter, plasma FSH concentrations started increasing and reached at the peak value between 150 and 210 min post GnRH administration and again, it declined gradually till 420 min post GnRH administration in experimental buffaloes. Plasma LH concentration started increasing within 30 min and reached at the peak value at 120 min post GnRH administration in experimental buffaloes. There was no change ($p=0.494$) of plasma AMH concentrations after GnRH challenge.

Plasma AMH profiles

In the present study, the parameter estimates of the best fitted cubic model are presented in Table 1. A cubic model was found to be the best fitted model that delineated the decline of plasma AMH with age. Plasma AMH profiles covering different ages in buffaloes are presented in Fig. 4.

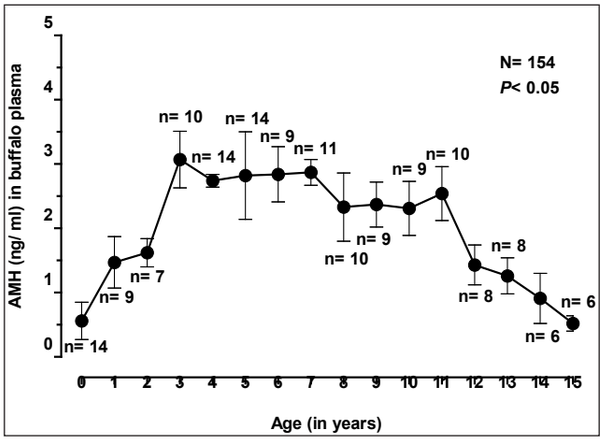

Fig. 4: Plasma AMH concentrations (ng/ ml) in buffaloes (n= 154) from 3 months to 15 years of ages. n represents the number of buffaloes at the particular age group

Table 1: Best fitted model for plasma AMH concentrations over time (age) in buffaloes

Animal species	Best fitted model	R-square	Fitted model
Buffalo	Cubic	0.897	$y = -0.316 + 1.051x - 0.101x^2 + 0.002x^3$

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

(time) in buffaloes. Plasma AMH concentration at 3 months of age was 0.56 ± 0.29 ng/ ml and thereafter it started increasing and reached at a higher level of 3.07 ± 0.44 ng/ ml at 3 years of age and then it remained same (≥ 2 ng/ ml) up to 11 years of age and then started progressively declining and reached at the level of 0.52 ± 0.12 ng/ ml at 15 years of age. Fig. 5 shows the superimpose of plasma AMH concentrations (ng/ ml) in buffaloes from 3 months to 15 years of ages with the best fitted cubic model curve.

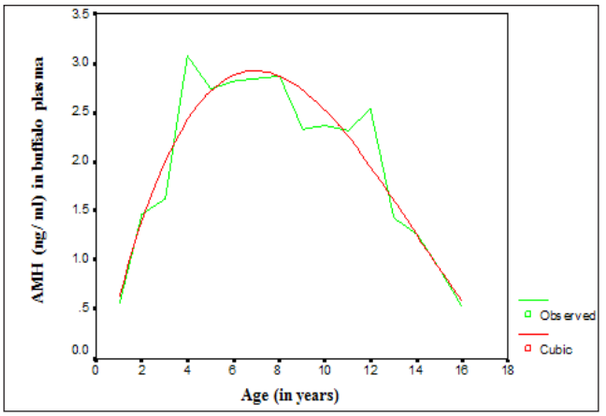

Fig. 5: Plasma AMH concentrations (ng/ ml) in buffaloes from 3 months to 15 years of ages is superimposed with the best fitted cubic model curve

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 Murrah buffaloes. The significant change ($p<0.01$) over time in pituitary secretions of FSH and LH after GnRH administration in buffaloes are quite similar to that of previous experiment on cattle (Fajersson *et al*., 1999). GnRH is a key regulator of reproductive functions, which triggers the release of FSH and LH from the pituitary gland that in turn regulate gonadal function and ovulation process (Schneider *et al*., 2006).

To the best of the current knowledge, no effect ($p>0.05$) of GnRH challenge on the ovary to produce AMH in Murrah buffaloes is the first report. The experiment showed that the plasma AMH level was independent of pituitary FSH and/ or LH action. In agreement, 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). Similarly, in conditions where FSH levels were suppressed, such as pregnancy, AMH levels remained constant (La Marca *et al*., 2005). Both in the human and bovine, AMH levels were almost independent of the phase of the ovarian cycle, explaining why a single AMH measurement has been usually sufficient (Rico *et al*., 2011). The 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). Thus, 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 Murrah buffaloes.

The present study determined the age-specific plasma AMH concentrations in Murrah buffaloes for the first time. 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 (Poomthavorn *et al*., 2009; Christiansen *et al*., 2016; Du *et al*., 2016). There was no information on age-related plasma AMH concentrations in buffaloes. The present study attempted to determine the peripheral AMH levels at different ages in Murrah buffaloes. The plasma AMH level has been just detectable at three months of age. Thereafter, the plasma AMH level increased at one year of age, peaking during the pubertal age and then showed a progressive decline throughout reproductive life and finally just detectable at fifteen year of age. A cubic model fitted best the decline of plasma AMH concentration with age. Circulating AMH concentration in young adult dairy heifers has been found to be a simple, reliable biomarker to predict productive herd life (time in a herd after birth of first calf) 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*., 2016). Plasma AMH concentration has been demonstrated 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). However, the size of ovarian reserve appears to be highly variable between individuals of similar age and between ages, but it is specific to each individual (Lahoz *et al*., 2014). Because AMH levels are strongly correlated with the ovarian follicular reserve,

plasma AMH levels may a good candidate for assessing individual reproductive status in buffaloes. The present study provides a first hand information on age-specific peripheral AMH levels in Murrah buffaloes.

CONCLUSION

The present study shows that GnRH stimulates pituitary to release FSH and LH into circulation, but it does not have any influence on the ovary to produce AMH in buffaloes. It indicates that the plasma AMH concentration is not influenced by hypothalamo-pituitary-gonadal axis or the gonadotropic status. Secondly, the present study determines the age-specific plasma AMH concentrations in buffaloes. A cubic model is the best fitted model that delineates the decline of plasma AMH level with age. A single AMH measurement in the plasma of buffaloes may open the possibility of using peripheral AMH concentration as a candidate endocrine marker for knowing the expected reproductive status of an individual buffalo.

ACKNOWLEDGEMENTS

The authors gratefully acknowledged the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi, India for funding the project (Grant number: BT/179/NE/TBP/2011). The authors would like to thank Dr. A.F. Parlow, National Hormone Peptide Program (NHPP), Harbor-UCLA Medical Centre, Carson, CA, USA for supplying ovine LH and ovine LH antiserum. We express our gratitude to the Director, ICAR Research Complex for North East Hill Region, Barapani, Meghalaya, India for the necessary supports and assistance during the study. The help and cooperation of the Technical and supporting staff of Livestock Farm, ICAR Research Complex for North East Hill Region, Tripura, India are duly acknowledged.

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