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Determination of anti-mullerian hormone in serum and ovarian histopathology of buffaloes

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Buffalo is one of the most important dairy animals concentrated largely in tropical and sub-tropical countries. They are the mainstay of dairy industry, especially in Asia and form the frail rural economy in many developing countries across the world. Anti-Mullerian hormone, also known as Mullerian Inhibiting Substance, is a glycoprotein of 140 kDa belonging to the transforming growth factor beta family that is expressed only in the gonads (Cate *et al*. 1986). Studies indicated that the antral follicle population may be of paramount importance to improve reproductive performance in cows. There is already an agreement that the antral follicle count (AFC; follicles >3 mm in diameter) is a highly variable trait among animals, but with high repeatability in the same individual. Thus, females can be classified into low, intermediate or high AFC (Morotti *et al.* 2015).

Anti-Mullerian hormone (AMH) is produced by granulosa cells of all primordial, primary, secondary follicles, as well as antral follicles up to 4 to 5 mm diameter and reflects the total number of healthy follicles within the ovaries. The function of AMH in females is to regulate or limit the recruitment of primordial follicles into folliculogenesis, by reducing the responsiveness of these follicles to follicle stimulating hormone. Anti-Mullerian hormone production decreases after antral stage follicles reach the 4 to 5 mm stage, allowing these follicles to regain responsiveness to follicle stimulating hormone and undergo final maturation (Visser *et al.* 2006). Moreover, circulating concentrations of Anti-Mullerian hormone (AMH) are positively associated with number of follicles or antral follicle count (AFC), ovarian function and fertility.

Anestrous is one of the most commonly occurring reproductive problems in cattle and buffalo in India, affecting livestock productivity and economics to a great extent. The condition may be associated with uterine pathology such as pyometra, fetal resorption, maceration and mummification. Expression of estrus is also influenced by seasonal changes, stress and aging. The infertility in buffaloes forced the farmers to sell their animals to butchers or slaughter house.

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The information regarding AMH concentration in cycle and anestrus buffaloes is scanty. Thus**,** the present research work was designed to correlate AMH concentration in serum and histopathological study of AFC in cyclic and anestrous buffaloes ovaries.

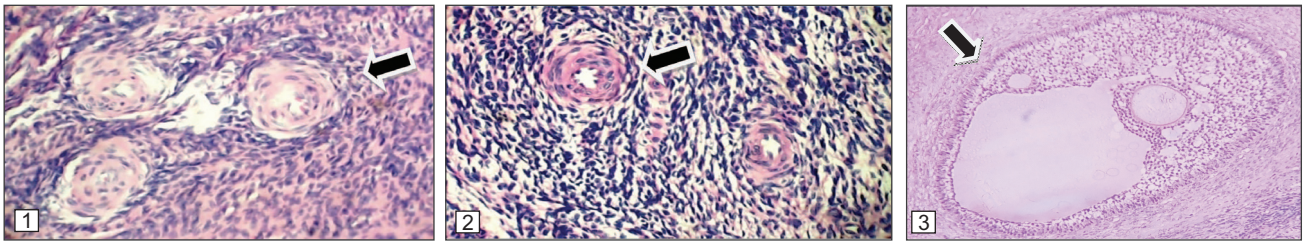
The buffalo ovaries were collected from slaughter house for histopathology. Total 30 pairs of ovary were collected. Blood samples were also collected before slaughtering the buffalo animals for estimation of AMH concentration. Ovaries collected in 10% Neutral Buffer Formalin were stained with routine Harris Hematoxyline and Eosin stain. Before processing, 10 serial sections of each pair of ovary were done for histopathological examinations. All the serial sections of the ovaries were processed as per the standard procedure. On the basis of histopathological examination, animals were categorized as cyclic and anestrus. Out of 30 pairs of ovary, 18 pairs were identified as anestrus and 12 as cyclic.

Follicles were classified as primordial if they contained an oocyte surrounded by a partial or a complete layer of squamous granulosa cells (Fig. 1). Primary follicles showed a single layer of cuboidal granulosa cells. Occasional follicles were observed as intermediate between primordial and primary and had both cuboidal and squamous granulosa cells. Follicles were classed as secondary if they possessed more than one layer of granulosa cells with no visible antrum. Early antral follicles possessed generally only one or two small areas of follicular fluid (antrum) whilst antral follicles possessed a single large antral space (Fig. 2). Pre-ovulatory follicles had a rim of cumulus cells surrounding the oocyte (Fig. 3).

Serum AMH concentration was evaluated by using bovine AMH ELISA kit, AL-114 (Ansh Labs, Webster, TX, USA). The sensitivity of the AMH assay was 11 pg/ml and intra-assay coefficients of variation (CV) was <5%.

The data were analyzed by Students T–Test using WASP-2 (Web Agri Stat Package), ICAR.

The means of primordial, antral and growing follicles in cyclic and anestrus buffaloes is presented in Table 1. The analysis of data revealed that, the growing follicles were significantly ($P<0.05$) higher in cyclic buffaloes than



Figs 1–3. **1.** Primordial ovarian follicles. **2.** Antral ovarian follicles. **3.** Pre-ovulatory follicles.

anestrus buffaloes, whereas, no significant difference between count of primordial and antral follicles in cyclic and anestrus buffaloes was observed in the present study. These findings are in accordance with Modina *et al.* (2013).

The significantly higher mean of growing follicles in cyclic buffaloes than anestrus buffaloes suggest that AMH is one of the factor determining the sensitivity of ovarian follicles for FSH and AMH is a dominant regulator of early follicle growth of growing follicles. The lower mean of growing follicles in anestrus buffaloes is due to AMH deficiency which inhibits the recruitment of primordial follicles into the pool of growing follicles and decrease the responsiveness of growing follicles to FSH (Visser *et al.* 2006).

The comparison of AMH concentration in cyclic and anestrous buffaloes is presented in Table 2. The analysis of data revealed significantly ($P<0.05$) higher AMH concentration in cyclic buffaloes than anestrous buffaloes. In the present study, the AMH concentration and growing follicles were higher in cyclic as compared to anestrous buffaloes, which is in accordance with Kevenaar *et al.* (2006) who reported that AMH is produced by growing follicles and observed significantly ($P<0.05$) lower AMH in anestrous mice.

Further they stated that, AMH decreases as the age increases, which supports the findings of the present study, because the growing follicles were higher in cyclic buffaloes, which might be one of the cause of high AMH in cyclic buffaloes as compared to the growing follicles of anestrous buffaloes. It can be assumed that the reduced AMH concentrations may be the consequence of decreased protein production by the mural cells lying immediately on the basal membrane of the follicles. In fact, it has been demonstrated that, in antral follicles, the outer layer of granulosa cells close to the theca is one of the regions of high AMH expression, where expression declines sharply in follicles undergoing atresia (Rico *et al.* 2009 and 2011). There is no possibility of pregnancy in anestrous buffaloes because their AMH concentration is very low in the present study. Rico *et al.* (2012) suggested that the animals having low AMH concentration below cut-off level (150 pg/ml) by determining single blood sample should be culled.

The AMH expression level of each granulosa cell depends on the stage of follicular development and more importantly, on its location inside the follicle. Among growing follicles, the population of largest follicles of the basal follicular development stage constitutes the pool of high AMH secreting and gonadotropin-responsive follicles. The size of this pool, i.e. the number of healthy follicles at this stage, varies between individuals but is quite steady within the animal over the long term and represents an intrinsic characteristic of each animal. The size of the follicular pool of AMH-secreting follicles and their responsiveness to intrafollicular and endocrine regulating factors such as bone morphogenetic proteins (BMP) and FSH, respectively, could both account for AMH endocrine levels. At each estrous cycle, the preovulatory and periovulatory FSH surges would inhibit AMH production by granulosa cells during the days following estrus, leading to a decrease in AMH endocrine levels. As a consequence, the optimal time for a blood test for estimating the size of the pool of gonadotropin-responsive follicles through measurement of AMH endocrine levels should take into account this dynamic profile for each cow to be tested (Rico *et al.* 2011).

Follicular count is positively correlated with AMH in the present study which is in agreement with the findings of Baldrighi *et al.* (2014) and Guerreiro *et al.* (2014). The glycoprotein AMH, which belongs to the transforming growth factor (TGF)-ß family, is only expressed in the gonads and is correlated to ovarian follicular development. AMH expression is observed in granulosa cells of growing preantral and antral follicles and it is described as a premature modulator of follicular growth by controlling premature depletion of the follicular reserve in ovaries (Durlinger *et al.* 2002).

Table 1. Mean±SE for primordial, antral and growing follicle counts of cyclic and anestrus buffaloes

Days	Buffaloes	Mean	t Stat	t table
Primordial	Cyclic Anestrus	0.42±0.07 0.49±0.02	–1.038^{NS}	2.048
Antral	Cyclic Anestrus	0.15±0.03 0.16±0.03	–0.079^{NS}	2.048
Growing	Cyclic Anestrus	4.47±0.88 0.41±0.06	5.687**	2.763

**Significant at 1% level, ^{NS}Non-significant difference.

Table 2. Mean±SE of AMH for cyclic and anestrous buffaloes

Animals	AMH (pg/ml)	t-Stat	t-table at 1 %
Cyclic Anoestrus	273.50±48.52 79.40±7.80	3.95**	2.87

**Significant at 1% level

Anti-Mullerian hormone is an endocrine marker and can predict the fertility of animal. The significantly lower AMH concentration and growing follicles in anestrous buffaloes indicated that there is no possibility of pregnancy in future. Whereas, cyclic buffaloes having significantly higher AMH concentration and growing follicles may remain pregnant if other infertility causes are corrected. The other infertility factors of pregnancy might be one of the reasons for bringing the animals for slaughter.

SUMMARY

Present study was undertaken to assess AMH concentration in serum and histopathological study of AFC in buffalo ovaries. Total 30 pair of ovary were collected in 10% Neutral Buffer Formalin. Blood samples were also collected before slaughtering the buffalo animals for estimation of AMH concentration. Before processing, 10 serial sections of each pair of ovary was done for histopathological examinations. All the serial sections of the ovaries were processed as per the standard procedure. On the basis of histopathological examinations and follicle count, animals were categorized as cyclic and anestrus. Out of 30 pairs of ovary, 18 pairs were identified as anestrus and 12 as cyclic. The mean number of primordial, antral and growing ovarian follicles count of cyclic buffaloes were 0.42±0.07, 0.15±0.03 and 4.47±0.03 and 0.49±0.02, 0.16±0.03 and 0.41±0.06 for anestrus buffaloes, respectively. Growing follicles were significantly higher in cyclic as compared to anestrous buffaloes. However, the difference of primordial and antral follicle count remained non-significant in cyclic and anestrous buffaloes. Whereas, AMH concentration in cyclic buffaloes (273.50±48.52) was significantly higher than anestrous buffaloes (79.40±7.80). Therefore, it is concluded that, anti-mullerian hormone is an endocrine marker and can predict the fertility of animal. Also, follicular count is positively correlated with AMH concentration.

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