



Evaluation of anti-Mullerian hormone, antral follicle count and progesterone concentration during estrous cycle in Murrah buffaloes

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Received: 7 December 2018; Accepted: 28 March 2019

ABSTRACT

An experiment was conducted to study the anti-Mullerian hormone (AMH) concentration, antral follicle count (AFC) and progesterone (P4) concentration during estrous cycle in Murrah buffaloes. Seven animals of 5–10 years were selected for the study. All the animals were synchronized as per the ovsync protocol and the pair of ovary of each animal was scanned by ultrasonography on 0, 3rd, 7th, 11th, 14th, 17th and 21st day of estrous cycle. Blood was collected on the same day to evaluate serum AMH and P4 concentration. The nonsignificant difference was observed in antral follicle count of 3–5 mm, 5–8 mm and total follicles count (> 3 mm), whereas, significantly lower mean values of >8 mm of antral follicles were recorded on the day of estrus (0.14 ± 0.14) as compared to 3rd, 7th, 11th, 14th, 17th and 21st day of estrous cycle. The significant individual variation was recorded in average mean of 3 - 5 mm and total antral follicle count (>3 mm). However, the difference for 5 - 8 mm and >8 mm was nonsignificant. The positive correlation of low and high antral follicle count was observed in the present study. Two animals of low antral follicle count remained non pregnant and two animals showed 3 follicular waves during estrous cycle. The nonsignificant difference was recorded in AMH concentration during estrous cycle, whereas, individual variation in AMH concentration differ significantly. The progesterone concentration showed significant increase and decrease in values according to the stages of estrous cycle.

Key words: Anti-Mullerian hormone, Antral follicle count, Buffaloes, Estrous cycle, Progesterone

Anti-Mullerian hormone (AMH), 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). The principal function of AMH is to induce regression of the Mullerian ducts during male sex differentiation. In the ovary, AMH is of key importance. It inhibits the recruitment of primordial follicles into the pool of growing follicles, and it decreases the responsiveness of growing follicles to follicle-stimulating hormone (Durlinger *et al.* 2002). Presently, AMH is the best endocrine marker of the ovarian follicular reserve in human, mouse, and recently the AMH is also found as a reliable endocrine marker of the population

of small antral gonadotropin responsive follicles in the cow (Rico *et al.* 2009). Over the past 10 years, attention has been focused on AMH in humans in the context of assisted reproductive technologies.

The AMH functions as a modulator of follicular development, preventing early follicular atresia and the depletion of the pre-antral follicular reserve (Monniaux *et al.* 2013). A major advantage of using AMH as a fertility marker is the small amount of variability throughout the many stages of reproductive and estrous cycles of cattle.

The information regarding AMH concentration during estrous cycle, in buffaloes is scanty. Therefore, the aim of the present research work is to evaluate antral follicle count (AFC), anti-Mullerian hormone (AMH) and progesterone (P4) concentration in cyclic Murrah buffaloes.

MATERIALS AND METHODS

Selection of animals: All the animals selected in this group were postpartum lactating buffaloes. Animals (9) were synchronized as per the protocol for the present study. All the animals were injected with the hormone GnRH and PGF2 α (Table 1) as per the standard ovsync protocol GnRH - PGF2 α - GnRH (G-P-G protocol). The buffaloes were kept on observation from -11th day onwards. The animal showing

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Table 1. Synchronization protocol for onset of estrous cycle in buffaloes

- 0 Day	- 7 th Day	- 9 th Day	-10 th Day onwards
GnRH - 5 ml (I/M)	PGF2 α - 2 ml I/M	GnRH- 5 ml (I/M)	Three times observation of estrus cycle

discharge from vagina and/or estrous behaviour was considered as estrus day, i.e. '0' day. Total 7 out of 9 buffaloes showed the sign of estrus, therefore 7 (seven) animals were continued for the research study.

Ultrasonography examination: All the buffaloes were examined by a real-time ultrasonography (SonoScape S2V - SN/3242090) with a probe of 7 to 8 MHz frequency, linear array by inserting per rectal. Pair of the ovaries of each animal was scanned by probe on 0, 3rd, 7th, 11th, 14th, 17th and 21st day of estrous cycle and images were saved for further analysis and record. The follicles of both the ovaries of each animal were counted during various stages of estrous cycle. The follicles >3 mm of diameter were counted and categorized as small (3–5 mm), medium (5–8 mm) and large (>8).

Blood collection: Blood was collected by jugular vein puncture on 0, 3rd, 7th, 11th, 14th, 17th and 21st day of estrous cycle in a vacutainer tube containing gel, for analysis of anti-Mullerian hormone (AMH) and progesterone (P4) concentration.

Artificial insemination: The artificial insemination (AI) of all the 7 animals was done on the occurrence of second estrous estrus cycle to know the fertility. Ultrasonography was performed in all the animals after 45 days as well per rectal examination was done after 2 months of insemination for confirmation of pregnancy.

Hormone assays: Serum AMH concentration was evaluated by using bovine AMHELISA kit (Ansh Labs, Webster, TX, USA). Whereas, the concentration of progesterone in serum samples was determined by using Beckman Coulter Company RIA kit (Ref. No. IM 1188, batch No. 171204C), Immunotech SAS, France. The assay procedures for evaluation of AMH and P4 were followed as per the directives of the manufacturer.

Management and feeding of buffaloes: All the animals were housed in a shed having concrete floor with asbestos

roof. Balanced concentrate mixture was offered to the experimental buffaloes as per the thumb rule. As the experimental animals were lactating buffaloes, 2 kg balanced concentrate mixture was offered for maintenance. The production requirement was calculated as for every 2 kg milk yield 1 kg balanced concentrate was offered, over and above the maintenance requirement. The fodder; green as well as dry including Para grass, Dharwad Hybrid Napier (DHN-6), paddy straw was offered *ad lib.* to meet out their dry matter requirement as well as to satisfy their bulk. The animals had free access to drink water, and all the animals were dewormed regularly.

Statistical analysis: The data was analyzed by CRD using WASP-2 (Web Agri Stat Package), ICAR, India.

RESULTS AND DISCUSSION

AFC during estrous cycle: The mean of small (3 - 5 mm), medium (5 - 8 mm), large (>8 mm) and total follicle count during estrous cycle are presented in Table 2.

The AFC of 3 - 5 mm did not differ significantly on 0, 3rd, 7th, 11th, 14th, 17th and 21st day, of the estrous cycle. In the present study, the number of small follicles of 3 to 5 mm were higher during metestrous (1 to 3 days), early diestrous (11th day) and follicular phase (17 to 21 days) and lower during early diestrous (5 to 10 days) and late diestrous phase (11 to 17 days), which is in accordance with Yilmaz *et al.* (2014).

The mean AFC of 5–8 mm were nonsignificant throughout the estrous cycle. The mean AFC for 5–8 mm was low with no specific trend of increase and decrease of follicles during estrous cycle. This may be due to the fact that number of medium follicles decreased or failed to grow during the three days before estrus when large follicles were growing and the dominant follicle inhibiting the growth of smaller follicles, which supports the findings of Warriach and Ahmad (2009) and that might be the reason of significantly ($P<0.05$) lower AFC of >8 mm on the day of estrus as compared to other days of estrous cycle, which remained nonsignificant.

The mean AFC of >8 mm was lowest on the day of estrus (day 0) which increased significantly ($P<0.05$) on day 3 and thereafter, it remained almost similar throughout estrous cycle till day 21 and did not differ significantly. The follicles above 8 mm were significantly ($P<0.05$) less during

Table 2. Mean $\pm$ SE of antral follicle count, P4 and AMH concentration during estrous cycle

Parameter	Day of estrous cycle						
	0	3 rd	7 th	11 th	14 th	17 th	21 st
3–5 mm	6.29 $\pm$ 0.52	4.71 $\pm$ 0.36	4.14 $\pm$ 0.67	5.00 $\pm$ 0.44	4.00 $\pm$ 0.31	4.71 $\pm$ 0.68	4.57 $\pm$ 0.61
5–8 mm	1.00 $\pm$ 0.22	1.71 $\pm$ 0.42	1.43 $\pm$ 0.48	2.14 $\pm$ 0.40	1.43 $\pm$ 0.30	1.71 $\pm$ 0.52	2.57 $\pm$ 0.37
>8 mm	0.14 $\pm$ 0.14 ^b	1.00 $\pm$ 0.22 ^a	1.14 $\pm$ 0.14 ^a	1.14 $\pm$ 0.26 ^a	0.86 $\pm$ 0.26 ^a	1.43 $\pm$ 0.30 ^a	1.28 $\pm$ 0.29 ^a
Total	7.43 $\pm$ 0.53	7.42 $\pm$ 0.65	6.71 $\pm$ 0.87	8.28 $\pm$ 0.68	6.29 $\pm$ 0.71	7.85 $\pm$ 0.51	8.42 $\pm$ 0.90
P4 (ng/ml)	0.47 $\pm$ 0.0 ^c	0.82 $\pm$ 0.02 ^c	1.83 $\pm$ 0.02 ^b	2.51 $\pm$ 0.21 ^a	2.84 $\pm$ 0.22 ^a	2.78 $\pm$ 0.20 ^a	0.48 $\pm$ 0.05 ^c
AMH (pg/ml)	517.86 $\pm$ 92.29	447.14 $\pm$ 92.03	380.00 $\pm$ 78.20	477.86 $\pm$ 94.35	415.00 $\pm$ 68.64	420.00 $\pm$ 70.45	487.14 $\pm$ 95.24

^{a,b,c}Values within a row with no common superscript differed significantly ($P<0.05$).

metestrus and follicular phase than early and late diestrus. It was observed that the population of these follicles was higher at the mid cycle period (between days 3 to 17), gradually decrease nonsignificantly in follicular phase of estrous cycle, which is similar to the observations reported by Yilmaz *et al.* (2014). They further stated that the presence of corpus luteum is responsible for atresia of the largest follicle due to negative regulation of LH pulse by secretion of progesterone.

The mean of total AFC did not differ significantly. The lowest count was recorded on day 7 and 14, followed by day 0, 3 and 17. The highest count was recorded on day 11 and 21. The above study of AFC of 3–5, 5–8 and >8 mm is in agreement with Warriach and Ahmad (2009) and Yilmaz *et al.* (2014) in buffaloes.

High variability and repeatability of maximum numbers of antral follicles >3 mm in diameter during follicular wave was recorded during estrous cycle in the present study, supports the findings of Burns *et al.* (2005). Two buffaloes in the present study showed 3 follicular waves and 5 buffaloes showed 2 follicular waves. The dominant follicle attained its maximum diameter on day 7 and 17 in 2 wave cycles (Figs 1, 2). Whereas, in 3 wave cycles, the second dominant follicle began to regress on day 19 when it was replaced by a third large dominant follicle which was the ovulatory follicle in all 3 wave cycles, which are comparable to the finding reported by Taylor and Rajamahendran (1991). Several studies had shown the prevalence of 2-wave follicular activity during an estrous cycle in cattle. This could be attributed due to the high incidence of the 2 or 3 waves of follicular activity might be based on presence of 2 or 3 peaks of gonadotrophic hormones, particularly FSH. The peaks of FSH were also related to lower estrogen concentration, which in turn depended on regression in follicular size (Ginther *et al.* 1996).

It is observed that significant ($P<0.05$) individual variation from animal to animal in total follicle count and 3–5 mm count was recorded (Table 3). Whereas, nonsignificant difference was recorded in 5–8 mm and <8 mm follicle count. The positive correlation of antral follicular count and fertility was observed in the present study. It was observed that buffaloes having low number of small follicles (3–5 mm) did not conceive and the buffaloes having significantly higher follicle counts

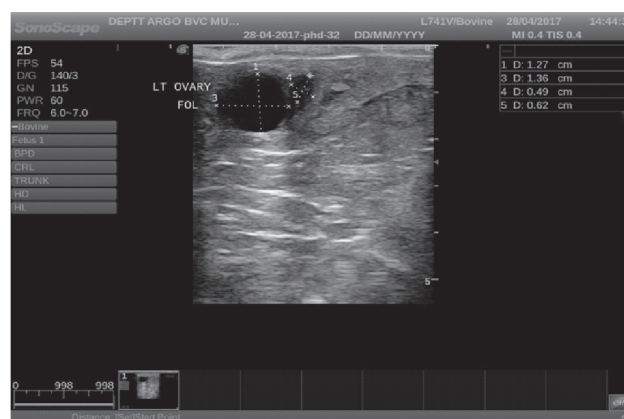


Fig. 1. Dominant follicle on day 7 of estrous cycle in cyclic buffaloes.

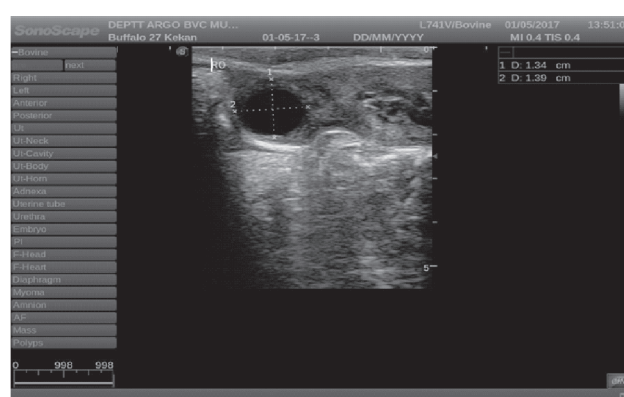


Fig. 2. Dominant follicle on day 17 of estrous cycle in cyclic buffaloes.

remained pregnant. Two buffaloes in the present study were not pregnant and 5 remained pregnant. This may probably be due to association between low AFC, enhanced FSH secretion, and decreased progesterone production may result in increased rates of embryo mortality (Mossa *et al.* 2012).

P4 concentration during estrous cycle: The lowest progesterone concentration was recorded on day 0 of estrous cycle, which increased nonsignificantly on day 3. From day 7 the progesterone concentration increased significantly ($P<0.01$) to a highest concentration on day 14. The P4 concentration remained almost similar on day 11, 14 and 17. Further it decreased again significantly ($P<0.01$) on day

Table 3. Average means $\pm$ SE of individual buffaloes for 3–5, 5–8, >8, total AFC and AMH concentration during estrous cycle

Buffalo tag No.	3 to 5mm	5 to 8 mm	>8 mm	Total follicle count (>3 mm)	AMH pg/ml	Pregnancy status
27	4.86 $\pm$ 0.51 ^b	1.86 $\pm$ 0.34	1.14 $\pm$ 0.26	7.86 $\pm$ 0.74 ^a	313.57 $\pm$ 15.30 ^d	Pregnant
30	6.71 $\pm$ 0.42 ^a	1.29 $\pm$ 0.42	0.57 $\pm$ 0.20	8.57 $\pm$ 0.57 ^a	822.14 $\pm$ 36.00 ^a	Pregnant
31	5.00 $\pm$ 0.38 ^b	2.00 $\pm$ 0.44	0.86 $\pm$ 0.26	7.86 $\pm$ 0.67 ^a	535.00 $\pm$ 41.59 ^c	Pregnant
32	5.00 $\pm$ 0.31 ^b	2.43 $\pm$ 0.48	1.29 $\pm$ 0.18	8.71 $\pm$ 0.57 ^a	300.00 $\pm$ 23.68 ^d	Pregnant
33	5.43 $\pm$ 0.37 ^b	1.57 $\pm$ 0.30	0.71 $\pm$ 0.18	7.71 $\pm$ 0.47 ^{ab}	637.85 $\pm$ 28.05 ^b	Pregnant
34	3.57 $\pm$ 0.30 ^c	1.00 $\pm$ 0.44	1.14 $\pm$ 0.26	5.71 $\pm$ 0.47 ^c	256.42 $\pm$ 7.94 ^d	Non pregnant
03	2.86 $\pm$ 0.26 ^c	1.86 $\pm$ 0.40	1.29 $\pm$ 0.42	6.00 $\pm$ 0.79 ^{bc}	280.00 $\pm$ 9.78 ^d	Non pregnant

Mean values within a column with no common superscript differed significantly (* $P<0.05$; ** $P<0.01$).

21. This is in accordance with Mondal *et al.* (2010) in buffaloes (Table 2).

The peripheral plasma progesterone profile in buffalo is very similar to that in cattle. Progesterone levels rise and fall in coincidence with the growth and regression of corpus luteum. Since CL is the source of progesterone in cycling buffalo. The peripheral progesterone concentrations are minimal on the day of estrus, rise to peak concentrations on days 13–15 of the cycle or even on day 17 before declining to basal levels at the onset of next estrus. Progesterone levels continue to increase in animals that conceive but drop 3 days before the next estrus in those that fail to conceive (Mondal *et al.* 2007).

AMH concentration during estrous cycle: The mean AMH concentration in buffaloes during estrous cycle differed nonsignificantly (Table 2). This nonsignificant difference with minor fluctuation throughout estrous cycle is in accordance with Akbarinejad *et al.* (2017) in cows.

The high AMH concentration on the day of estrus and then decreasing on day 3 and 7 of estrous cycle may be due to inhibition of AMH production by FSH at granulosa cell level but enhanced by bone morphogenetic proteins. The expression of AMH within the follicle is dependent on the stage of follicular development. At the ovarian level, the size of the pool of small antral growing follicles determined ovarian AMH production. At the endocrine level, AMH followed a specific dynamic profile during the estrous cycle, which occurred independently of the follicular waves of terminal follicular development (Rico *et al.* 2011).

The nonsignificant decrease in AMH concentration following estrus is not associated with concomitant changes in the numbers of follicles detected by ovarian ultrasonography, but it could result from the inhibiting action of FSH upon AMH production by granulosa cells of the AMH secreting follicles in the basal follicular growth stage. Further, whether there is any direct or indirect effect of LH and GH or prolactin on AMH production should also be investigated.

Further, the decrease in AMH concentration during the days following estrus may be in response to pre- and peri-ovulatory FSH surges. The high FSH concentration resulting from 2 consecutive FSH discharges may reduce AMH production by the granulosa cells of the small antral, high AMH-producing follicles (Monniaux *et al.* 2013). Bovine granulosa cells from cows with low antral follicle counts are less sensitive to FSH, in terms of FSH induction of *in vitro* estradiol production, compared with granulosa cells from cows with high antral follicle counts, which may be due to expression of lower FSH-receptor mRNA in granulosa cells from cows with low versus high antral follicle counts (Scheetz 2010).

AMH and pregnancy: Highly significant ($P < 0.01$) individual variation in the mean concentration of AMH was recorded in the present study. It ranged from 256.42 ± 7.94 to 822.14 ± 36.00 pg/ml (Table 3). The variation of AMH in individual animals in the present study is in accordance with Monniaux *et al.* (2013) in cows.

The positive association between AMH and fertility was observed in the present study wherein, 5 buffaloes remained pregnant and 2 buffaloes were non pregnant. It was observed that, the buffalo no. 34 and 03 had low AMH concentration of 256.42 ± 7.94 and 280.00 ± 9.78 , respectively, which remained non pregnant and differed significantly ($P < 0.01$) to the AMH concentration of pregnant buffaloes. The positive association between AMH and fertility is similar to the observations reported by Jimenez-Krassel *et al.* (2015). Riberio *et al.* (2014) stated that pregnancy maintenance was affected by AMH. Cows with low AMH had greater risk of pregnancy loss between day 30 and 65 of gestation than cows with intermediate or high AMH.

Correlation of AMH, AFC and pregnancy status in cyclic buffaloes: The relationship between serum AMH and AFC of 3 to 5 mm was positively correlated during estrous cycle. The magnitude of correlation coefficient was $r = 0.80$, and highly significant ($P < 0.01$). This is in accordance with Center *et al.* (2018) in cows.

According to Pfeiffer *et al.* (2014), the relationship among gonadotropins, AFC, and AMH is not clear. The capacity of granulosa cells to produce AMH depends on the concentration of FSH. Increased and decreased concentrations of FSH produce variation in the production of AMH by granulosa cells. Increased concentrations of FSH have been correlated with decreased production of AMH. The decline in production of AMH could possibly be attributed to luteinization of granulosa cells. The effects of gonadotropins on regulation of AMH require further elucidation. Decreased concentrations of FSH have been correlated with an increased production of AMH concomitant with an increased production of estradiol. Follicle diameter did not differ between the natural or synchronized estrous cycles, and no correlation between diameter and concentration of AMH was established. They concluded that the variation in concentrations of AMH specifically during the emergence and regression of follicular waves requires further establishment.

The positive association between AFC, AMH and fertility was observed in individual buffaloes of the present study, wherein 5 buffaloes remained pregnant and 2 buffaloes were non pregnant. It was observed that, the buffaloes with significantly low AMH concentration (256.42 ± 7.94 and 280.00 ± 9.78 pg/ml), and AFC (3.57 ± 0.30 and 2.86 ± 0.26 ng/ml), respectively, remained non pregnant as compared to the AMH and AFC mean values of pregnant buffaloes. Jimenez-Krassel *et al.* (2015) also found positive correlation between AFC, AMH and fertility which correlate the findings of the present study.

The antral follicle count is positively associated with AMH concentrations levels and is a good indicator for high, medium, and low AFC. Antral follicle count could possibly be associated with the fertility predictor's made using AFC without actually analyzing AFC in cattle, a producer can simply look at AMH levels prior to ovulation (Scheetz 2010). He further stated that concentrations of AMH did not change with AFC groups during the 6 to 8 days prior to

ovulation, but were ~6 to 2 fold greater in animals in the high and medium AFC groups compared with animals in the low AFC group.

The positive correlation between anti-Mullerian hormone, antral follicle count of 3–5 mm and fertility was observed. The buffaloes with high AMH and high AFC were pregnant as compared to low AMH and low AFC. The buffaloes which are having 2 or 3 follicular waves did not affect the conception rate. Whereas, significant individual variation were recorded in AMH, P4; small and large AFC. Thus, it is concluded that, the AMH concentration is a reliable phenotypic marker to predict the number of healthy follicles and oocytes in ovaries and predict the future potential of buffaloes.

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