ULTRASONOGRAPHIC MONITORING AND FOLLICULAR DYNAMICS IN PERI-PUBERTAL ACYCLIC MURRAH BUFFALO HEIFERS (*BUBALUS BUBALIS*) IN DIFFERENT SEASONS

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ABSTRACT

The peripubertal anestrous Murrah buffalo heifers (*Bubalus bubalis*) during summer (Jun-Aug; n=14) and winter season (Dec-Feb; n=12) were selected at random from herd and the ovaries of the experimental animals were scanned for a period of 20 days on every alternate day and in these animals wave and its duration, the number of follicles, size of follicles, growth and regression profiles of dominant and subordinate follicles were recorded and the follicular dynamics was monitored. The follicles continue to grow and regress without ovulation during anestrous period in the peripubertal heifers. The maximum size of DF remained smaller (9.29 ± 0.18 mm) in anovulatory follicles. At puberty, the CL formed after 1st ovulation was of short duration and the second ovulation occurred within 6-10 days interval. A significantly more number of total antral follicles in both the ovaries were observed during winter season as compared to those during summer season.

Key words: Dominant follicle, growth rate, regression phase, wave duration, ultrasonography

Introduction

India's largest livestock resources among the world play an important role in uplifting the socio economic status of country's rural population. Low reproductive efficiency in buffalo remains a major economic problem throughout the world but with still a higher incidence in India (Kumar *et al.*, 2009) and this low reproductive efficiency has been attributed to some inherent problems like late maturity, silent oestrus, poor expression of oestrus and inactive ovaries (Cockrill, 1980; Madan, 1988). The application of ultrasonography in bovine reproduction has made it possible to monitor the follicular population during estrous cycle (Pierson and Ginther, 1987a, b, 1988), anoestrus period (Henao *et al*., 2000), postpartum period (Ruiz-Cortes and Olivera-Angel, 1999) and during hormonal treatment (Pierson and Adam, 1999). Furthermore, transrectal ultrasonic imaging provides a mean for repeated, direct, non-invasive monitoring and measuring of follicles regardless of their depth in the ovary (Johnson *et al.,* 1994; Garcia *et al.,* 1999). Baruselli *et al*. (1997) demonstrated that two wave follicular growth is more common in buffaloes during breeding season. Kumar *et al*. (2001) found no effect of early pregnancy on wave pattern of follicular development in buffaloes, which occurred in two or three waves during one cycle length, however, dominant follicle in pregnant animals tended to be smaller than in the non-pregnant buffaloes. Predominance of two wave follicular activity has also been reported in buffaloes during winter season (Manik *et al*., 1998). However, follicular dynamics in buffalo heifers during summer and winter seasons has not been fully explored, therefore, the present study was designed to monitor the ovarian follicular activities and to study follicular turnover pattern in peripubertal murrah buffalo heifers during summer and winter seasons.

Materials and Methods

Study location and animals

The present study was conducted at ICAR-Central Institute for Research on Buffaloes, Hisar during summer (Jun-Aug) and winter season (Dec-Feb) on 26 peri-pubertal anestrous Murrah buffalo heifers (*Bubalus bubalis*). All these heifers were between age groups of 30-36 months weighing 300-340 kg body weight having good body condition of 2.75 -3.0. From the herd, 26 anestrous heifers for each season were selected at random after a proper ultrasound examination of the ovaries for this study for two seasons *i.e*. Summer (n=14) and winter (n=12).

Housing, feeding and management

Experimental buffalo heifers were housed in half-walled pucca sheds with asbestos sheet roof. Heifers were let loose daily for pasture grazing for 3-4 hr. In addition, the animals were stall-fed with adequate quantity of green fodder and wheat-straw. The concentrate feed, supplemented with mineral mixture and common salt prepared in the institute, was also provided to the animals. Drinking water was available *ad libitum*. Animals were protected from the inclement weather and given water shower bath daily during summer season.

Schedule for ultrasonographic monitoring

Summer season

The ovaries of the experimental animals were scanned for a period of 20 days on every alternate day *i.e*., Day 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20; (Day 0 -day of start of ultrasonographic examination in experimental animals) and in these animals the number of follicles, size of follicles, growth and regression profiles of dominant and subordinate follicles were recorded and the follicular dynamics was monitored as follow:

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a. Number of dominant and subordinate waves.
b. Wave duration of dominant and subordinate follicles (days).
c. Maximum size of anovulatory dominant and subordinate follicles (mm).
d. Growth rate of dominant and subordinate follicles (mm/day).
e. Regression rate of dominant and subordinate follicles (mm/day).
f. Growth phase duration of dominant and subordinate follicles (days).
g. Regression phase duration of dominant and subordinate follicles (days).
h. Total number of large follicles (≥9 mm) (Alvarez *et al*., 1994).
i. Maximum size of ovulatory dominant follicles (mm).
j. Growth rate of ovulatory dominant follicles (mm/day).

Winter season

During winter season similar to that of summer season 12 acyclic heifers from the herd were selected at random and all these experimental animals were subjected to ultrasonographic monitoring for a period of 20 days and the follicular dynamic parameters were monitored as summer season.

Ultrasonographic monitoring for follicular dynamic before and during treatment protocol

Ovarian follicular changes were monitored in all experimental buffalo heifers with a real time B-mode ultrasound scanner (Just Vision 200–Model SSA-320A, Toshiba, Japan) equipped with a convex array multi frequency transducer using frequency of 7.0 MHz. After properly restraining the animal in a chute, without use of any tranquilizing agent, well lubricated transducer was inserted together with hand in the rectum after evacuating the faecal material. For orientation, the transducer was moved along the dorsal surface of the reproductive tract and then moved laterally to examine the ovaries. During examination, individual ovary and the transducer were held in the same hand. Ovaries were scanned by turn, visualizing the structures on each ovary by sliding the probe from medial to the lateral aspect of the ovary and recording all structures for their location and size.

Total follicular population on an ovary was recorded by noting down each follicle observed during scanning of individual ovary through its entire surface, from medial to lateral aspect on any given day. Smallest appreciable follicles over 2 mm in diameter were recorded. The positions and sizes of follicles (≥3 mm) were traced at each scanning and the exact location of follicles was recorded. The follicles were categorized as small, medium and large having size of ≥2 mm to 4.9 mm, ≥ 5 - 8.9 mm and ≥ 9 mm, respectively.

Statistical analysis

The data obtained in the present study were subjected to Analysis of Variance and students T-test analysis (Snedecor and Cochran, 1994) to draw scientific inferences. Characteristics associated with ovarian follicular development were examined with General Linear Model Procedures in the statistical software package for Windows Version 9.0.1 (SPSS Inc. Chicago, IL).

Results and Discussion

Follicular wave and wave duration

In the 14 heifers subjected to ultrasonographic examination during the summer season, a total of 10 complete dominant follicular waves were observed where as during the winter season (n=12) a slightly higher number (14) of dominant follicular waves were observed (Table 1). The mean duration of dominant follicular waves in summer season was slightly longer (17.60±0.58 days) compared to that of winter season (16.0±0.58 days, Table 1). Similarly, the number of subordinate follicular waves was higher during winter season as compared to the summer season (42 v/s 24). The wave duration of DF and the SF was almost similar during both the seasons (Table 1).

Size and numbers of follicles

In this study, it was observed that the maximum size of anovulatory DF (9.29±0.18mm) and SF (6.31±0.21 mm) during the summer season was significantly higher than that observed during the winter season (8.65±0.28 mm and 5.74±0.12 mm). The size of the DF observed during both the seasons was also significantly higher than the size of SF recorded during summer (n=14) as well as winter season (n=12, Table 2); and further observed that the size of DF which ovulated during winter season was significantly higher (11.4±0.3 mm) compared to that of the anovulatory DF (8.65 ± 0.28 mm, Table 2).

During the summer season the mean number of antral follicles (>2 mm Diameter) observed in the ovaries of peripubertal heifers on any given day of ultrasonographic monitoring was 24.34±0.36 and ranged from 22.50±1.69 to 26.21 ±1.26. However, the mean number of follicles during winter season was 36.67±0.42 (range from 34.71±1.27 to 38.86±1.81, Fig. 1).

The total number of antral follicles observed on any day was recorded to be significantly higher during the winter season as compared to the summer season (Table 3). It was observed that in 40.26% of the observations recorded by ultrasonographic examination, large follicles were present and the number of large follicles ranged from 0-2 on the given day.

Growth rate and growth phase duration of follicles

The growth rate of the SF was recorded as slightly higher but statistically non-significant during both summer as well as winter season compared to that of the DF. However, the growth rate of ovulatory DF (0.83±0.03 mm/day) was higher than growth rate of anovulatory DF (0.71±0.06 mm/day) during winter season, but it was statistically non-significant. Similarly, the growth rate of DF as well as SF was more during the winter season as compared to that of during summer season (Table 4).

The growth phase duration of DF and SF was almost similar during both the seasons. However, when growth phase duration of DF was compared with that of SF, the growth phase duration of DF was significantly higher during summer (9.85±0.52 v/s 4.77±0.54 days) as well as winter season (9.64±0.45 v/s 4.32 ±0.32 days), respectively (Table 4).

Regression rate and regression phase duration of DF and SF

The regression rate of the anovulatory DF and SF was

Table 1: Wave and Wave duration of dominant and subordinate follicles

Sr. No.	Parameters	N/n	Summer (Mean±SEM)	N/n	Winter (Mean±SEM)
1.	Total number of complete waves of dominant follicle during 20 days in 14 heifers		10		14
2.	Total number of complete waves of Sub ordinate follicle during 20 days in 14 heifers		24		42
3.	Wave duration of DF(days)	10/14	17.60±0.58^a	14/12	16.0±0.92^a
4.	Wave duration of SF(days)	24/14	8.67±0.84^b	42 /12	8.33±0.48^b
5.	Total ovulatory wave of DF	0*	0*	5	9

N- Number of waves, n- numbers of animals; *No ovulatory wave observed in experimental animals during summer season, while in winter season in one animal ovulation of 1 DF and in 4 animals ovulation of Two DF occurred. Mean bearing different superscripts (a, b) differ significantly in a column (P<0.05).

Table 2: The mean size of follicles

Sr. No.	Parameters	N/n	Summer (Mean ±SEM)	N/n	Winter (Mean ±SEM)
1.	Maximum Size of Anovulatory DF (mm)	41	9.29±0.18^a	32	8.65±0.28^b
2	Maximum Size of Anovulatory SF (mm)	33	6.31±0.21^c	48	5.74±0.12^d
3.	Maximum Size of Ovulatory DF (mm)	0*	-	9	11.4±0.30^e

N- Number of follicles, n- numbers of animals; *No ovulatory follicle was observed in experimental animals during summer season, while in winter season in one animal ovulation of 1 DF and in 4 animals ovulation of Two DF occurred. Means bearing different superscripts (a, b, c, d and e) differ significantly in a column (P<0.05).

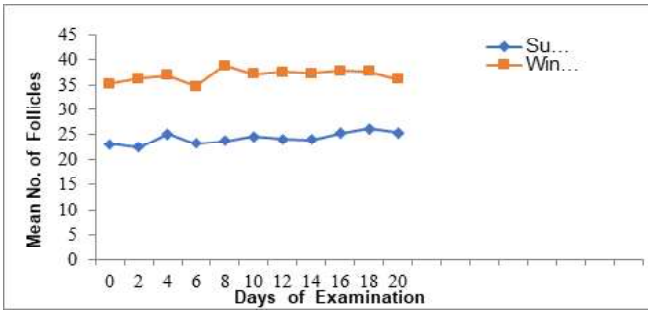

Fig. 1: Mean number of follicles on both ovaries

Table 3: Mean number of total follicles in the heifers

Parameter	Day	n	Summer Mean ±SEM	n	Winter Mean ±SEM
Number of total follicles (on any given day of ultrasonographic monitoring)	0	14	23.14±0.75^a	12	35.21±1.43^b
	2	14	22.50±1.69^a	12	36.21±1.29^b
	4	14	25.21±1.33^a	12	36.93±1.56^b
	6	14	23.29±1.32^a	12	34.71±1.27^b
	8	14	23.71±1.17^a	12	38.86±1.81^b
	10	14	24.71±1.11^a	12	37.07±1.76^b
	12	14	24.21±0.96^a	12	37.57±1.23^b
	14	14	24.0±1.35^a	12	37.29±1.13^b
	6	14	25.29±1.43^a	12	37.29±1.51^b
	18	14	26.21±1.26^a	12	37.64±1.28^b
	20	14	25.43±1.21^a	12	36.07±1.1^b

n-Number of animals during summer and winter seasons, Means bearing different superscripts (a, b) differ significantly in a column (P<0.05).

Table 4: Growth rate and growth phase duration of DF and SF

Sr. No.	Parameters	N	Summer (Mean±SEM)	N	Winter (Mean±SEM)
1.	Growth rate of Anovulatory DF (mm/day)	35	0.65±0.05^a	28	0.71±0.06^a
2	Growth rate of SF (mm/day)	32	0.74±0.06^a	47	0.79±0.06^a
3.	Growth phase duration of DF(days)	26	9.85±0.52^a	33	9.64±0.45^a
4	Growth phase duration of SF (days)	26	4.77±0.54^b	45	4.32±0.32^b
5.	Growth rate of ovulatory DF	0*	0*	9	0.83±0.03^a

N-Number of follicles, (n= 14 in summer; n=12 in winter; n- numbers of animals), *No ovulatory follicle was observed in experimental animals during summer season, while in winter season in one animal ovulation of 1 DF and in 4 animals ovulation of Two DF occurred. Means bearing different superscripts (a, b) differ significantly in a column (P<0.05).

slightly higher during the winter season compared to that of summer but the regression phase duration of DF during summer was significantly higher (9.39±0.57 days) than that recorded during the winter season (7.24±0.56 days). The result indicates that the DF took a longer time for its regression during summer as compared to the winter season (Table 5). When the regression phase duration of the DF was compared with that of SF, it was observed that duration was higher for the DF during the summer (9.39±0.57 v/s 4.64±0.45 days) and the winter season (7.24±0.56 v/s 4.44±0.34 days).The regression phase duration of SF was almost similar during the both seasons.

It is difficult to assess the cyclicity by per rectal palpation therefore, ultrasonography is considered as more reliable technique for detection of ovarian structures in buffaloes (Rajamahendran *et al.,* 1994). With the advent of ultrasound it has become possible to closely monitor the development of antral follicles in which oocyte resides before its eventual ovulation close to oestrus. All the animals in the present experiment were monitored ultrasonically and confirmed as anestrous by the absence of cyclic corpus luteum.

The pattern and dynamics of follicular growth during

Table 5: Regression rate and regression phase duration of DF and SF of heifers

Sr. No.	Parameters	N/n	Summer (Mean±SEM)	N/n	Winter (Mean±SEM)
1.	Regression rate of Anovulatory DF (mm/day)	30/14	0.63±0.05^a	21/12	0.73±0.07^a
2	Regression rate of Anovulatory SF (mm/day)	34/14	0.78±0.07^a	50/12	0.79±0.05^a
3	Regression phase duration DF(days)	23/14	9.39±0.57^a	21/12	7.24±0.56^b
4	Regression phase duration SF(days)	31/14	4.64±0.45^c	45/12	4.44±0.34^c

N-Number of follicles, n- numbers of animals. Means bearing different superscripts (a, b and c) differ significantly in a column (P<0.05).

Ultrasonograph 1: Showing ovary with small multiple follicles

Ultrasonograph 2: Showing ovary with large dominant follicle

estrous cycle have been extensively investigated in cattle (Lucy *et al.,* 1992; Ginther *et al.,* 1997). However, reports on progression of follicular growth are very limited in buffaloes (Baruselli *et al.,* 1997; Manik *et al.,* 1998).

The dominant follicles of anovulatory waves showed two phases of growth The results of the present study demonstrated that ovarian follicles developed in a wave like fashion in peripubertal Murrah buffalo heifers during anestrous period as previously observed in prepubertal Murrah buffalo heifers (Sharma *et al.*, 2010), 5-9 months old prepubertal Mediterranean Italian buffalo calves (Presicce *et al.,* 2003).In the present study, the ultrasonographic examination revealed that during this period, follicles continues to grow without ovulation of largest DF of wave. The largest DF of the wave subsequently undergoes atresia. A new follicular wave then emerges with several follicles growing at the same time but only one of these subsequently developed to be the largest DF. Our study is in accordance with the report of Sharma *et al.* (2010) who reported wave pattern of follicular growth in prepubertal Murrah heifers. In the present study, the mean wave duration during summer was slightly longer as compared to that during winter seasons (17.60±0.58 v/s 16.0±0.92 days). This indicates that the follicles grow and regress at a faster rate in winter season than summer. Similar to that of the present study, Sharma *et al.* (2010) observed wave duration of 15.14±1.08 days in prepubertal heifers during summer season.

During ultrasonographic monitoring of follicular dynamics, 5/14 heifers became cyclic during winter season compared to none (0/14) during summer season. The result further revealed that the duration of ovulatory wave following first ovulation was 6-10 days. A similar short waves lasting <10 days (6.1±1.2) in 5-9 months old prepubertal Mediterranean Italian buffalo calves was also reported by Presicce *et al.* (2003). After first ovulation, a new wave started and the second ovulation occurred after a short interval of 6-10 days in our study. However, Presicce *et al.* (2005a) observed a longer ovulatory interval (days) between first postpartum ovulation and second ovulation in primiparous (18.3±1.2) and pluriparous (14±2.7) buffaloes during months of increased day length. This longer ovulation interval observed by Presicce *et al.* (2005b) may be due to different parity status of experimental animals.

The CL formed after first ovulation in these heifers was of short duration. A number of other studies also recorded short cycle following first ovulation (Presicce *et al.,* 2003; Sharma *et al.,* 2012). The diameter of DF was significantly larger than that of subordinate's follicles both during summer and winter seasons. The size of the dominant follicles during winter season was smaller (8.65±0.28 mm) as compared to those during summer (9.29±0.018 mm). This is because of the fact that large dominant follicles observed during of winter seasons (11.4±0.30 mm) undergone ovulation, therefore the size was not included for the purpose of calculation. Because of this reason, size of anovulatory follicles during winter season was recorded as significantly lower than those during summer season. Spicer *et al*. (1986) observed that large antral follicles are present during early postpartum but they do not ovulate. They further observed that this may be due to inability of these large follicles to stimulate preovulatory gonadotrophins surges because of production of less amount of estradiol.

The mean maximum diameter of DF reported in the present study was smaller as compared to the values for adult buffaloes (Taneja *et al.,* 1996; Baruselli *et al*., 1997; Manik *et al.,* 1998) and larger than those reported (8.4±1.2 mm) in 5-9 months old prepubertal Mediterranean Italian buffalo calves (Presicce *et al.,* 2003). However, the results are in accordance with the findings of Sharma *et al.* (2010) in prepubertal Murrah buffalo heifers. This suggests that the size of DF increases over time till it reaches a value equivalent to the ovulatory size (11.4±0.30 mm) as observed in some heifers (n=5) during winter season. Similar to that of our study a reduced size in prepubertal bovine compared to that of pubertal animals has also been reported (Bergfeld *et al.,* 1994, Evans *et al.,* 1994). Presicce *et al.* (2005a) observed a higher diameter of first postpartum ovulating follicle in primiparous (13.5±0.8 mm) and pluriparous (14.1±0.4 mm) buffaloes during months of increased day length. This higher diameter observed by Presicce *et al.* (2005b) may be due to different parity status of experimental animals.

The growth phase duration of DF was significantly higher

(9.85±0.52, summer; 9.64±0.45 winter) than that of SF (4.77±0.54, summer; 4.32±0.32, winter and it is similar to that of the findings of Sharma *et al.* (2010). However, Presicce *et al.* (2005a) observed a slightly higher growth phase duration of first postpartum ovulating follicle in primiparous (11.7±1.5 days) but a similar growth phase duration in pluriparous (9.5±0.8 days) buffaloes.

The growth rate of ovulatory DF was higher (0.83±0.03 mm/day) compared to that of anovulatory DF (0.71±0.06 mm/day). The growth rate of DF and SF in our study did not differ significantly and also no significant difference was observed either during summer or winter season. The DF and SF emerges almost at the same time with similar growth rate however, SF cease to grow after some time and became atretic. A similar growth rate (0.79±0.1 mm/day) of SF was also reported by Presicce *et al.* (2003) in prepubertal Italian buffalo heifers, however, he reported a much higher growth rate of DF (1.08±0.2 mm/day) in these heifers. Similarly, Sharma *et al.* (2010) also reported faster growth rate of DF (0.58±0.04mm/day) as compared to that of SF (0.43±0.03 mm/day) in prepubertal Murrah buffalo heifers. However, Presicce *et al.* (2005a) observed a higher growth rate of first postpartum ovulating follicle in primiparous (0.95±0.18 mm/day) and pluriparous (1.07±0.07 mm/d) buffaloes during months of increased day length. The present study support the findings of Ginther *et al.* (1989a) that the subordinate follicles of each follicular wave cease to grow after a few days and that a new wave does not appear, while a dominant follicle is in its growth phase or at the beginning of its static phase. There was no difference in size between the future dominant follicle and the largest subordinate follicle at the time of wave emergence.

The regression phase duration of SF was observed significantly lower than the DF. Furthermore, regression phase of DF was shorter during winter (7.24±0.56 days) as compared to those during summer (9.39± 0.57 days). However, there was no significant difference between the two seasons with respect to regression phase of the SF. The regression rate of DF was slightly lower than SF. The finding indicate that SF grew and regressed at a faster rate compared DF but with a non significant difference. Presicce *et al.* (2003) also reported a similar pattern of regression rate in prepubertal Italian buffalo heifers. The regression of the DF is followed by emergence of a new follicular wave in which a variables number of small follicles emerged in a cohort and one of these subsequently developed to be the largest DF. In contrast to the present study, a high value of regression rate was observed in Murrah buffalo during oestrous cycle (Baruselli *et al.,* 1997).

There was little variation between the numbers of follicles in the different waves at the time of their emergence. This is in agreement with studies in buffaloes (Baruselli *et al.,* 1997) and cattle (Ginther *et al.,* 1989b), and suggests that the number of follicles recruited depends on the individual animal. This shows a fluctuation caused by periodic selection of growing follicles from the available pool. There was no significant difference with respect to day of examination during each season. The mean number of total antral follicles of >2 mm differed significantly (P< 0.05) during summer and winter season indicating emergence of constant number of follicles at any day during anestrous condition. In the present study the mean number of follicles on both ovaries observed were 24.34±0.36 (ranged from 22.5±1.69 to 26.21±1.26) during summer season and 36.67±0.42 (ranged from 34.71±1.27 to 38.86±1.81) during winter season. Danell *et al.,* (1987) reported that the ovaries of cyclic and non-cyclic Surti buffalo heifers possessed 46.3 versus 67.8 follicles (>1 mm), and 16.8 versus 23.6 follicles (> 2 mm), respectively. Kumar (2010) studied follicular dynamics in different groups of postpartum buffaloes before treatment *i.e*. in anestrous condition and reported that the average number of follicles on both ovaries ranged between 7.50±0.50 to 9.33±1.69 during anestrous condition. The difference may be due to measurement of all follicles of ≥2mm in our study in contrast to measurement of ≥3 mm in that study. In cattle also, dynamic follicular activity continued during anestrous period before first postpartum ovulation (Henao *et al.,* 2000). Kastelic *et al*. (1990) also provided indirect evidence that the two components of functional dominance, the ability to ovulate and the ability to suppress the emergence of a new follicular wave are closely linked in time, and they emphasized that size alone is not a good indicator of functional dominance. Therefore, from the present study it is concluded that:

i. The follicles continue to grow and regress without ovulation during anestrous period in the peripubertal heifers. The maximum size of DF remained smaller (9.29 ± 0.18 mm) in anovulatory follicles.
ii. At puberty, the CL formed after 1st ovulation was of short duration and the second ovulation occurred within 6-10 days interval.
iii. A significantly more number of total antral follicles in both the ovaries were observed during winter season as compared to those during summer season.
iv. The growth rate as well as regression rate of follicles was more during winter season, however, the growth phase duration and regression phase duration of both DF and SF was more during summer season.

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