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Distribution, morphometric evaluation and steroidogenic profile of ovarian follicles in cyclic and acyclic buffalo (*Bubalus bubalis*)

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ABSTRACT

Acyclicity in buffaloes represents as one of the most prevalent disorders affecting fertility and productivity. It commonly occurs during the postpartum period or under conditions of nutritional, environmental, or metabolic stress, ultimately increasing the number of days open, veterinary interventions, and culling rates. Understanding the distribution pattern of follicles and their functional status is, therefore, essential for developing targeted reproductive interventions. The present study was conducted at ICAR-Indian Veterinary Research Institute, during 2021-22. Paired ovaries were collected from healthy adult buffaloes (n=75) at slaughter. Ovaries were classified as cyclic or acyclic based on the presence of a CL. Surface follicles were enumerated and categorized into small (<5 mm), medium (5–10 mm), and large (>10 mm). Follicular fluid from large follicles was aspirated for estimation of estradiol (E_2), progesterone (P_4), androstenedione (A_4) and testosterone (T) concentration, and follicles were retrospectively classified as active (P_4:E_2 <1), intermediate (P_4:E_2 1–10), or atretic (P_4:E_2 >10). Acyclic buffaloes exhibited a greater proportion of ovaries with only small follicles (36.36%), compared with cyclic animals (9.43%), whereas only large follicles occurred predominantly in cyclic buffaloes. Cyclic buffalo ovaries also showed stage-dependent shifts in follicle distribution. Large follicles from cyclic and acyclic buffaloes displayed comparable steroidogenic patterns, with progressive increases in P_4 and decreases in E_2 from active to atretic categories. Further, the E_2:A_4 ratio was also significantly greater in the active follicle than in the atretic follicle. Thus, evaluating follicles using the E_2:P_4 and E_2:A_4 ratios provided a more accurate indication of follicular functional status than classification based solely on follicle size and cycle stage.

Keywords: Acyclic, Buffalo, Cyclic stage, Follicle, Ovary, Steroid profile

Despite substantial economic importance of buffaloes, reproductive performance remains a major constraint, mostly arises from a complex interaction of inherent genetic and physiological limitations along with external factors such as environmental stressors, nutritional status, and management practices (Nanda *et al.* 2003). Among the reproductive health issues, ovarian acyclicity represents as a primary contributor to infertility in water buffalo (Das and Khan, 2010; Zicarelli, 2011). Studies have consistently shown that acyclic buffaloes exhibit distinct deviations in follicular dynamics compared to their cyclic counterparts. In cyclic buffalo, the ovarian cortex houses all developing follicles, which undergo a multi-stage maturation process from primordial to preovulatory states. However, in acyclic buffaloes, there is typically a reduced population of larger, mature follicles (Honparkhe *et al.* 2022; Khan *et al.* 2011, 2012). Further, the cyclic buffaloes produce a higher number of good-quality oocytes compared to acyclic buffaloes indicating compromised ability of acyclic buffalo ovaries to support the growth and maturation of follicles to an ovulatory stage (Mehmood *et al.* 2024). The steroid profile in the follicular fluid (FF) and systemic circulation is significantly altered during ovarian acyclicity. In acyclic buffaloes, follicular fluid concentrations of estradiol (E_2), are significantly lower across different follicle sizes compared to cyclic animals (Honparkhe *et al.* 2022; Khan *et al.* 2012). Conversely, progesterone (P_4) levels in the follicular fluid of acyclic buffaloes may be higher in smaller follicles, but fail to increase adequately in larger follicles, indicating impaired steroidogenesis (Honparkhe *et al.* 2022; Khan *et al.* 2012). The observed endocrine disturbances in acyclic buffaloes are also reflected in their plasma profiles, with acyclic animals generally showing lower circulating levels of E_2 and P_4 compared to cyclic buffaloes (Patel *et al.* 2020). These hormonal imbalances further impair follicular growth and steroid hormone production (Jerome *et al.* 2017; Warriach *et al.* 2015). Therefore, a thorough understanding of how follicles are distributed, their morphometric measurements, and hormone-producing abilities is vital

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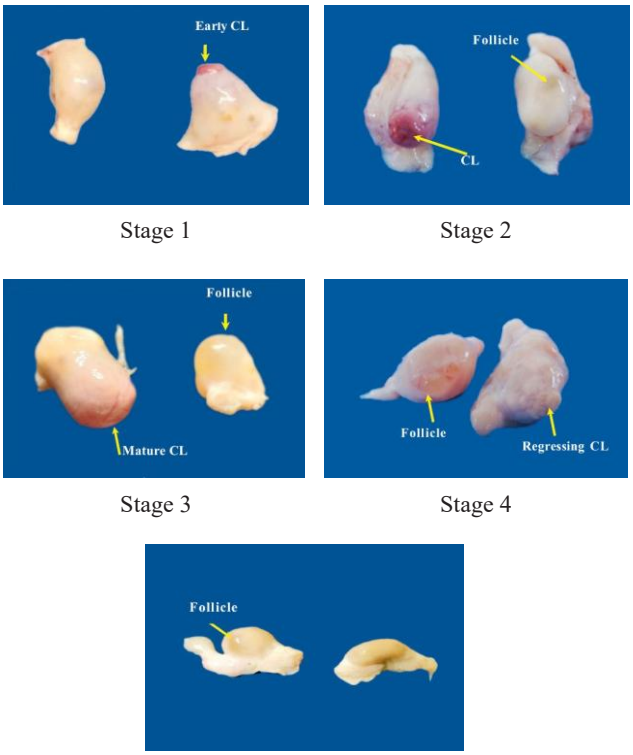
for distinguishing true acyclicity from undetected estrus and creating specific nutritional, husbandry, and endocrine treatment strategies. The present study was designed to examine the morphometric distribution characteristics of ovarian follicles along with steroid hormone concentrations in follicular fluid in cyclic and acyclic buffaloes.

MATERIALS AND METHODS

Collection of experimental material: The present study was conducted at ICAR-Indian Veterinary Research Institute, Izatnagar during 2021-22. The experiment was based on the slaughter-house specimens; hence ethical approval for animal experiment was not required. Paired ovaries (n=80) were collected from apparently healthy adult buffaloes (*Bubalus bubalis*) immediately after slaughter at Marya Frozen Agro Pvt. Ltd., Bareilly. The ovaries were separated from the attached adnexa and shifted to the laboratory in ice. Out of these, five pairs of ovaries were discarded due to the presence of cysts, ovaro-bursal adhesion, and dermoid cysts. Based on the presence of the CL in either of the ovaries (Fig.1), the samples were broadly classified into cyclic and acyclic, respectively (El-Wishy, 2007; Khan *et al.* 2011; Sheikh, 2018). The CL was enucleated from the ovary, and the stage of the estrous cycle was determined by gross appearance of CL (colour, consistency, and vascularization) and size of follicles (Jaglan, 2008; Mishra *et al.* 2018). In the cyclic buffaloes, the gross appearance of CL changed from bright red and soft in consistency in early stage (stage 1) to reddish brown to orange in colour with growing blood vessels in early-mid stage (stage 2). At late-mid stage (stage 3), CL colour turned to tan to orange brown in colour with a compact consistency, which became creamy white to brown creamy in colour with firm consistency at late stage (stage 4). Acyclic ovaries were typically smaller in size and smooth in consistency, with a variable number and sizes of surface follicles (Fig.1).



Stage 1 Stage 2

Stage 3 Stage 4

Acyclic

Fig. 1. The bubaline ovaries at different stages of the estrous cycle and at acyclic stage

Follicular morphometry: The ovaries with appreciable follicular activity were considered for the present study. The number and size of surface follicles were recorded for each pair of ovaries. The follicles of <5mm were considered as small, 5-10 mm as medium, and >10 mm as large in size. The follicular fluid was aspirated from the largest follicle using a 5 ml disposable syringe with an 18 G needle. After centrifugation at 3000 rpm for 10 min, the follicular fluid supernatant was stored at −20°C for estimation of estradiol (E_2), progesterone (P_4), androstenedione (A_4), and testosterone (T) concentration.

Estimation of steroid hormones in follicular fluid : Follicular fluid steroid hormones such as E_2, P_4, A_4 and T concentration were determined using solid phase I^{125} commercial radioimmunoassay kits (Beckman Coulter, Immunotech, France). The intra-assay coefficients of variation for P_4, E_2, A_4, and T were 8.1%, 14.4%, 10.1%, 10.6%, respectively, and inter-assay coefficients of variation were 8.7%, 14.5%, 14.6% and 19%, respectively. Based on the proportion of P_4:E_2, the follicles were classified into active, P_4:E_2 <1 (n =16), intermediate, P_4:E_2 between 1-10 (n=18), and atretic, P_4:E_2 >10 (n=11), retrospectively (Grimes *et al.* 1987; Ireland and Roche, 1983).

Statistical analysis: The proportion of follicles in cyclic and acyclic categories was compared using Z test. The follicular fluid E_2, P_4, A_4, and T concentration at each category of follicles was compared using one-way ANOVA with Tukey's post-hoc test. The difference of mean values for all data analysed with $p < 0.05$ was considered significant, whereas $p < 0.10$ was considered as a tendency towards significance.

RESULTS AND DISCUSSION

The present study provided a comprehensive evaluation of ovarian follicular distribution, morphometry, and steroidogenic milieu in cyclic and acyclic buffaloes, offering important insights regarding ovarian acyclicity. The classification of ovaries based on the presence or absence of a CL revealed clear gross morphological differences between cyclic and acyclic buffaloes. Cyclic ovaries exhibited distinct luteal stages characterized by progressive changes in color, consistency, and vascularity of the CL, consistent with the earlier reports. In contrast, acyclic ovaries were smaller, smoother, and lacked CL, indicating ovarian inactivity or failure of ovulation. These findings supported the use of gross luteal characteristics as a reliable indicator of ovarian cyclicity in slaughterhouse-derived samples.

Table 1. Distribution patterns of surface follicles in the ovaries of cyclic and acyclic buffalo

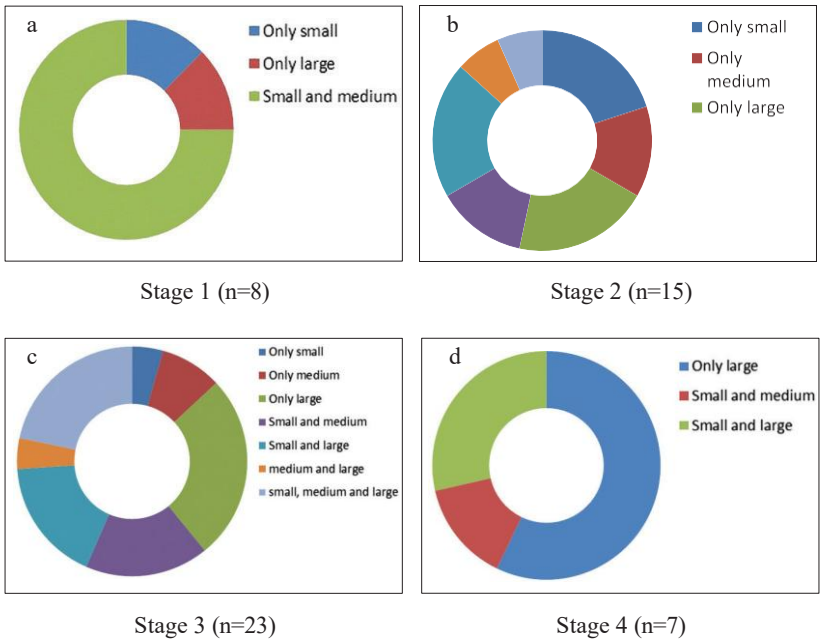
Types of Follicle	Cyclic (N = 53)	Acyclic (N = 22)
Only small	5 (9.43)	8 (36.37) *
Only medium	4 (7.55)	1 (4.55)
Only large	14 (26.42) *	1 (4.55)
Small and medium	13 (24.53)	4 (18.18)
Small and large	9 (16.98)	4 (18.18)
Medium and large	2 (3.77)	1 (4.55)
Small, medium and large	6 (11.32)	3 (13.54)

Follicles were categorized based on size. Small <5 mm, medium >5 to <10 mm, large >10 mm. N= number of animals. Values in parentheses indicate per cent of the animal. Values with an asterisk indicate a significant difference at $p<0.05$.

The distribution of small (<5mm), medium (5-10 mm), and large follicles (>10 mm) in the ovaries of cyclic and acyclic buffaloes is presented in Table 1. The proportion of ovaries with only small follicle were significantly ($P<0.05$) higher in the acyclic buffaloes compared to the cyclic group (36.4 vs. 9.4 %). Ovaries with a large follicle (>10 mm) were, however, observed in a higher proportion of cyclic buffaloes than the acyclic (26.4 vs. 4.6 %) buffaloes. A higher proportion of acyclic ovaries contained exclusively small follicles, whereas cyclic ovaries more frequently harbored large (>10 mm) follicles, indicating impaired follicular progression in acyclic buffaloes. This observation was consistent with earlier reports demonstrating that follicular recruitment was largely preserved in acyclic buffaloes, but subsequent selection of a dominant, ovulatory follicle was compromised (Baruselli *et al.* 1997, Das and Khan, 2010, El-Wishy, 2007). However, the distribution of ovaries with small and medium, small and large, or small, medium and large types of follicles was comparable irrespective of the cyclic status in the buffalo.

Among the cyclic buffaloes, approximately 72% (38 out of 53) were at mid-luteal stage (Stage 2 and 3), about 15% buffaloes were at stage 1, and 13 % were at stage 4. In stage 1, 75% buffaloes had small and medium-sized follicles, whereas in stage 2, the distribution of follicles was highly variable consists of about 20% belong to only large and small and large types. Similarly, in stage 3, around 26% buffaloes had only large follicles and 21.8% had small, medium and large types of follicles in the ovaries. In stage 4, 57.1% buffaloes had only large follicles, and 28.6% had small and large type follicles (Fig. 2). The predominance of large follicles during the late luteal stages in cyclic buffaloes reflected normal follicular wave turnover, supporting the reliability of gross luteal morphology for estrous cycle staging in slaughterhouse-derived ovaries.

However, there was no significant difference in the size among the large follicles irrespective of functional status; the size ranged from 10.25±0.37 to 11.36±0.86 mm (Table 2). Among the acyclic buffaloes, the follicular fluid P_4 and E_2 concentrations were similar irrespective of the functional status from active to atretic. On the contrary, the cyclic buffaloes had a significantly greater P_4 concentration in the atretic follicles, followed by intermediate than the active follicles ($p=0.01$). However, the E_2 concentration tended to be higher in active and intermediate type than those of atretic categories ($p=0.06$) of follicles in cyclic buffaloes. Functional classification based on the P_4:E_2 ratio revealed a clear steroidogenic gradient from active to atretic follicles. Active follicles were characterized by



Stage 1 (n=8) Stage 2 (n=15)

Stage 3 (n=23) Stage 4 (n=7)

Fig. 2. Pie diagram showing the distribution of follicles in different cyclic stages of buffalo. a. Stage 1, b. Stage 2, c. Stage 3, and d. Stage 4

Table 2. Comparison of steroid hormone profile in the follicular fluid between cyclic (N=34) and acyclic buffaloes (N=11) in relation to the functional status of the follicle

Attributes	Cyclic Pattern	Active (n1=2, n2=14)	Intermediate (n1=5, n2=13)	Atretic (n1=4, n2=7)	P value
Follicle size (mm)	Acyclic	11 ± 0.41	11.33 ± 0.47	10.25 ± 0.37	0.92
	Cyclic	10.54 ± 0.63	10.75 ± 0.57	11.36 ± 0.86	0.72
P_4 (ng/mL)	Acyclic	5.40 ± 1.79	27.59 ± 16.59	56.60 ± 33.29	0.34
	Cyclic	3.71 ± 1.28[a]	28.12 ± 8.78[b]	95.89 ± 31[c]	0.01
E_2 (ng/mL)	Acyclic	8.83 ± 0.71	5.21 ± 2.61	2.91 ± 0.24	0.26
	Cyclic	13.57 ± 3.98	10.21 ± 2.54	0.72 ± 0.52	0.06
A_4 (ng/mL)	Acyclic	5.3 ± 1.2	33.13 ± 13.42	19.75 ± 3.56	0.22
	Cyclic	15.35 ± 4.34	18.36 ± 9.97	47.41 ± 18.46	0.10
T (ng/mL)	Acyclic	4.95 ± 0.13	6.51 ± 1.81	6.62 ± 0.58	0.66
	Cyclic	15.57 ± 8.63	7.25 ± 2.92	10.52 ± 1.82	0.62
E_2: P_4 ratio	Acyclic	4.01 ± 1.20[a]	0.25 ± 0.07[b]	0.02 ± 0.01[c]	<0.01
	Cyclic	5.66 ± 2.27[a]	0.49 ± 0.06[b]	0.02 ± 0.0[c]	0.03
A_4: P_4 ratio	Acyclic	1.58 ± 0.30	2.85 ± 1.55	4.35 ± 3.07	0.64
	Cyclic	5.65 ± 2.10	1.18 ± 0.5	0.98 ± 0.54	0.05
E_2: A_4 ratio	Acyclic	2.14 ± 0.35[a]	0.94 ± 0.60[a]	0.17 ± 0.13[b]	0.04
	Cyclic	2.67 ± 0.92	3.02 ± 0.86	0.02 ± 0.01	0.09
E_2: T ratio	Acyclic	1.77 ± 0.1[a]	0.63 ± 0.24[b]	0.47 ± 0.3[b]	0.03
	Cyclic	1.92 ± 0.66	2.45 ± 0.74	0.08 ± 0.05	0.10

Functional status of the follicles was categorized based on the progesterone (P_4) and estradiol (E_2) ratios for active <1; intermediate 1-10, and atretic >10. Values bearing different superscripts (a-c) in a row for each attribute differ significantly at $P < 0.05$. N= number of follicles, n_1= number of follicles in acyclic, and n_2= number of follicles in the cyclic buffalo.

E_2 dominance and low P_4 concentrations, whereas atretic follicles exhibited significantly elevated P_4 and diminished E_2 concentration. This shift was consistent with classical descriptions of follicular atresia, wherein granulosa cells undergo luteinization-like changes accompanied by loss of aromatase activity (Grimes *et al.* 1987; Ireland and Roche, 1983). The progressive decline in the E_2:P_4 ratio across follicle categories observed in both cyclic and acyclic buffaloes underscored the conserved nature of steroidogenic remodelling during follicular degeneration. However, the ratio of E_2:P_4 was significantly different in the active, intermediate, and atretic categories of follicles in acyclic ($p<0.01$) and cyclic ($p<0.05$) buffaloes. Similar observations have been reported previously, wherein follicles from acyclic buffaloes retained the capacity to synthesize steroid hormones despite their failure to culminate in ovulation (Khan *et al.* 2012). These findings suggest that ovarian acyclicity in buffalo is not primarily attributable to intrinsic follicular steroidogenic incompetence but may reflect disruptions at the level of hypothalamic pituitary regulation, metabolic signalling, or gonadotropin pulsatility.

Interestingly, the A_4 and T concentrations were similar irrespective of follicular status in the cyclic and acyclic buffaloes, suggesting that theca cell androgen synthesis remains relatively stable during follicular growth and atresia. Comparable findings have been reported in buffalo follicles, where androgen availability was maintained even as E_2 synthesis declined (Khan *et al.* 2011). The significantly reduced E_2:A_4 and E_2:T ratios in atretic follicles further indicated that impaired aromatization, rather than decreased androgen substrate supply, is a key determinant of follicular atresia in buffalo. Similarly, the E_2:A_4 ratio of active follicles was also significantly greater than that of atretic follicles in acyclic buffaloes ($p=0.04$), and it tended to be significant in cyclic buffaloes ($p=0.09$). The A_4:P_4 concentration ratio was similar irrespective of the functional status of the follicles in acyclic buffaloes, while in the cyclic buffaloes, the ratio tended to be significantly higher in cyclic active follicles than the intermediate and atretic types ($p=0.05$). On the other hand, the E_2:T ratio was significantly greater in the acyclic active follicles compared to the intermediate and atretic follicles. In the cyclic buffalo, it remained similar across functional states of the follicles.

The absence of major steroidogenic differences between cyclic and acyclic buffalo follicles has important implications for field-level reproductive management. The presence of hormonally active follicles in acyclic ovaries suggested that a proportion of buffaloes diagnosed as acyclic may, in fact, have the potential to be induced into estrus, if provided optimal LH support or experienced anovulation. Previous studies have also emphasized that weak estrus expression and suboptimal heat detection

substantially contribute to apparent infertility in buffalo herds (Roy and Prakash, 2009; Warriach *et al.* 2015). Therefore, reliance solely on ovarian morphology may lead to misclassification of reproductive status. Collectively, these findings emphasized that ovarian acyclicity in buffaloes is not merely a consequence of arrested follicular growth but also involves subtle endocrine dysregulation that prevents the establishment of E_2-dominant, ovulatory follicles. The presence of steroidogenically comparable follicles in cyclic and acyclic ovaries highlighted the importance of accurate estrus detection and differentiation between true acyclicity and silent estrus. From a practical standpoint, this underscores the potential value of targeted endocrine, nutritional, and management interventions aimed at restoring hypothalamic-pituitary-ovarian axis functionality rather than focusing solely on the ovary.

The present findings reinforce the concept that ovarian acyclicity in buffaloes is characterized more by dysregulation of follicular dominance and endocrine signalling than by absolute failure of follicular growth or steroidogenesis. Cyclic buffaloes possess a higher number of large, hormonally active follicles with efficient steroidogenesis, whereas acyclic buffaloes exhibit impaired follicular growth, reduced E_2 and P_4 secretion, and increased follicular atresia. These structural and hormonal alterations underlie reduced fertility in acyclic buffaloes. Therefore, functional assessment of follicles based on intra-follicular steroid ratios provides a robust framework for distinguishing true ovarian inactivity from undetected estrus. Such insights are essential for the development of targeted endocrine and management interventions aimed at improving fertility in buffalo production systems.

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