



Relationship of external genitalia measurement with antral follicular count and estrus induction response in pre-pubertal Murrah buffalo heifers

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ABSTRACT

This study was designed to deduce the inter-relationship between external genitalia measurement, antral follicular count (AFC) and body weight (BW) and evaluate estrus induction rate following the administration of clomiphene citrate in pre-pubertal Murrah buffalo heifers. For this study, twenty pre-pubertal heifers (age: 30–36 months; BW: 367±8.6 kg) were selected and their external genitalia along with their relative rima length (RRL) and vulval width (RVW) and AFC using trans-rectal ultrasonography were recorded. Two groups were studied: control (n=9) and treatment (n=11). Treated heifers were given clomiphene citrate at 300 mg/heifer orally for 9 days and in control group, placebo (copper sulphate tablets) were given and onset of cyclicity as well as estrus signs was detected at the end of the treatment. All the heifers showed signs of estrus, but none ovulated. Also, AFC differed between heifers with low and high RRL (6.5±0.44 vs 8.6±0.67) and RVW (6.53±0.47 vs 8.16±0.70). Likewise, BW was correlated with RRL and AFC with BW, RRL and RVW. Non-significant difference was observed between the two groups with respect to dominant and sub-ordinate I and II follicles' size. Following clomiphene citrate supplementation, crestar treatment was administered to both groups resulting in pregnancy in control animals (n=2) and treated animals (n=6). In conclusion, this study showed that BW, external genital measurements and AFC are correlated and oral administration of clomiphene citrate failed to induce fertile estrus in pre-pubertal Murrah buffalo heifers.

Keywords: Antral follicular count, Buffalo, Clomiphene citrate, Heifers

Antral follicular count (AFC), is considered as an important measurement of fertility in cattle and buffalo (Alward *et al.* 2023, Ireland *et al.* 2008) as ovarian follicular development is an easy characteristic feature for phenotype classification. AFC influences the efficiency of reproductive biotechnologies and reproductive performance in cattle and buffaloes. This count has high variation among animals as well as high repeatability within individuals monitored by ultrasonography exam during follicular waves (Kavya *et al.* 2019, de Lima *et al.* 2020). In recent studies, based on AFC, bovine females were grouped based on the AFC and used for reproductive selection (Baldrighi *et al.* 2014, Batista *et al.* 2014). It has been reported that higher AFC is positively correlated with characteristics of female fertility in buffaloes (Baldrighi *et al.* 2014, Kavya *et al.* 2017), but needs further detailed studies on these lines.

Onset of puberty is an important factor influencing reproduction and lifetime productivity of a female, including buffaloes (Day and Anderson 1998). Use of estradiol compounds have used in cattle and buffalo for estrus induction and synchronization (Kankal *et al.* 2008).

In particular, clomiphene citrate has been tried as it causes depletion of estrogen receptors at pituitary and hypothalamic level, inhibiting the negative feedback exerted by estrogen normally (Fouda and Sayed 2011). Considering this, GnRH secretion is increased which in turn stimulates pituitary for FSH production and potentiates growth and maturation of follicles (Rehman *et al.* 2014). In cattle and buffaloes, clomiphene citrate is a non-steroidal synthetic hormone with estrogenic effect, which has been used as an initial therapy for ovulation induction (El-Sherry *et al.* 2011).

Considering its structural resemblances to estrogen, it binds competitively with nuclear receptors of estrogen and decreases the negative feedback of estrogen and enhances pituitary gonadotropin hormones release (Pakrasi and Kumar 2001, Sovino *et al.* 2002). Thus, the present study was conducted to test the hypothesis whether external genital measurements are associated with AFC as well as to elucidate whether Crestar® treatment following oral administration of clomiphene citrate induces fertile estrus in pre-pubertal Murrah buffalo heifers.

MATERIALS AND METHODS

Study location and animals: This study was conducted at ICAR-Central Institute for Research on Murrah buffaloes (ICAR-CIRB), Hisar, located at 212 m above mean sea

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level at 29.17°N latitude and 75.72°E longitude with an average annual rainfall of 459 mm.

Pre-pubertal Murrah buffalo heifers (n=20) having age of 30–36 months were selected. This study was approved by ICAR-CIRB supervisory committee. Heifers were not found in estrous activity before the start of experiment ascertained by transrectal ultrasonography at 10 days interval. Heifers were housed at the Livestock Farm, ICAR-CIRB, Hisar, had free access to water and were fed green fodder and wheat straw as per feeding standards (ICAR 2013). The mean age of treated group [(34.17±1.83) months] differed non-significantly compared with control group [(33.00±2.10) months]. Similarly mean weight of both groups [(378.33±8.76) kg vs (369.33±8.29) kg] was also not significantly different and housing and management was optimum. Rectal palpation was conducted to evaluate uterine development, ovarian size and presence of corpus luteum was ascertained using transrectal ultrasonography.

Experimental 1: Measurement of external genitalia and estimation of body weight: The external genital, i.e. rima length and vulval width was measured using vernier calipers as done in cattle (Mesquita *et al.* 2016). To adjust the external genitalia measurements to animal size, RL and VW were divided by the body weight (BW) and termed relative rima length (RRL) and relative VW (RVW), respectively. Based on RRL and RVW, the following groups were formed for analyses: low = 1.90 and high > 1.90; low = 1.30 and high > 1.30, respectively.

Experimental 2: Estrus induction using clomiphene citrate: Out of these selected Murrah buffalo heifers, eleven heifers were assigned to treatment group and other nine to control group. Identification tags were allotted to each heifer. Treated heifers were given clomiphene citrate at 300 mg/heifer orally (Clofci tablet, UNIM Pharma Lab Pvt. Ltd. Gujarat, India) for 9 days, at the same time animals of control group were given placebo Copper Sulphate tablets. Following treatment, all the heifers were treated with Crestar® (Intervet, Boxmeer, The Netherlands) on day 10 followed by PMSG (day 16) and on day 17, Crestar® was removed to evaluate the priming of clomiphene citrate for estrus induction. All heifers showing estrus were inseminated and pregnancy diagnosis was carried out after 30 days post insemination by trans-rectal ultrasonography.

Trans-rectal ultrasound examination and detection of estrus: Trans-rectal ultrasound examination was carried out in both groups on day 0 (start of experiment), 4 and 9 during the experiment for ascertaining the cyclicity of the study heifers. The study animals were detected for estrus at the end of the treatment of estrus signs (estrus discharge, frequent urination and uterine tone). Onset of cyclicity was determined by the presence of estrus signs following the development of corpus luteum post ovulation during ultrasound examination. Antral follicular count was estimated as per earlier report (Kavya *et al.* 2017).

Statistical analysis: Difference in different categories of follicular profile and the number of animals showing onset of estrus was calculated using study T-test and Chi-square

test, respectively and results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

To the best of our knowledge, there is first report of deducing association between external genital with AFC in pre-pubertal Murrah buffalo heifers. The external genital measurement, i.e. RRL and RVW of the different follicular count group is shown in Table 1. It is evident that AFC differed ($p=0.0236$) significantly between the two RRL groups (low and high). Similarly, with respect to RLW, AFC showed a trend ($p=0.0715$) between the two AFC group (low and high). It was interesting to note that BW was correlated with RRL ($r=-0.56$; $p=0.012$) and RVW ($r=-0.4$; $p=0.09$). Similarly, RRL was significantly correlated with RVW ($r=0.71$; $p=0.001$). Also, AFC was correlated with BW ($r=0.53$; $p=0.019$), RRL ($r=-0.47$; $p=0.039$) and RVW ($r=-0.46$; $p=0.048$) (Table 2). Though, no report in the literature demonstrating similar association for comparisons in buffaloes, nonetheless, such studies have shown that genital dimensions were related to ovarian reserve in cows (Mesquita *et al.* 2016, Vasconcelos *et al.* 2020). Study in Tabapuã breed showed higher AFC with higher RL and VW relative indices (Maculan *et al.* 2018). Through these data it is evident that external genitalia measurement indices can convey precise predictions related to fertility traits. It is also notable that relative size indices were associated with AFC and in turn fertility which is evident from our study. In our study, BW was correlated with RRL as well as AFC, significantly. In Zebu/Holstein cows, it was deduced that AFC was greater in animals having greater VW as well as RL. These data correlate that external genitalia measurements are also associated with AFC, as long as, their relation to female size (Mesquita

Table 1. External genital measurement (relative rima length and relative vulvae width) of different antral follicular count group

Parameter	Group	AFC	p-value
RRL	Low	8.6±0.67 (n=5)	0.0236
	High	6.5±0.44 (n=15)	
RVW	Low	8.16±0.70 (n=7)	0.0715
	High	6.53±0.47 (n=13)	

RRL, Relative Rima Length; RVW, Relative Vulvae Width; AFC, Antral follicular count.

Table 2. Correlation between body weight, relative rima length, relative vulvae width and antral follicular count

Parameter	Correlation (r^2)	p-value
BW	RRL (-0.56)	0.012
	RVW (-0.4)	0.09
RRL	RVW (0.71)	0.001
	BW (0.53)	0.019
AFC	RRL (-0.47)	0.039
	RVW (-0.46)	0.048

BW, Body weight; RRL, Relative rima length; RVW, Relative vulvae width; AFC, Antral follicular count.

Table 3. Fertility status of the in-study heifers

Parameters	Group		Chi-square; p-value
	Control (n=9) (%)	Treatment (n=11) (%)	
Estrus signs			
Vulval swelling (n)	0/9 (0 %)	11/11 (100 %)	16.164; <0.0001
b. Uterine tone (n)	0/9 (0 %)	11/11 (100 %)	16.164; <0.0001
c. Estrus discharge (n)	0/9 (0 %)	11/11 (100 %)	16.164; <0.0001
d. No. of animals pregnant (n)	2/9 (22.22 %)	6/11 (54.54 %)	1.768; 0.183
Follicle count (n)	p- value		
	Control		Treatment
	Day 0	Day 4	Day 9
>10 mm	2	2	4
5-9 mm	16	17	18
1-4 mm	48	46	66

Table 4. Follicular profile in study groups

Follicular profile	Group		p-value		
	Control (n=9)	Treatment (n=11)	Treatment	Time	Treatment*time
Dominant follicle size (mm)	7.97±0.35	7.86±0.75	0.8137	0.0466	0.0868
Sub-ordinate follicle I size (mm)	5.01±0.37	5.12±0.31	0.7871	0.3933	0.1346
Sub-ordinate follicle II size (mm)	3.47±0.23	3.62±0.11	0.6697	0.6788	0.2488

et al. 2016). In this study, RRL was more strongly associated with AFC than RVW. This approach has proven to be useful and relatively feasible which may be applied in the selection of cows with higher AFC under field conditions.

In the present study, all the treated heifers showed signs of estrus, viz. vulval swelling, uterine tone and estrus discharge ($p < 0.0001$); but none of the treated heifers in both groups ovulated. Furthermore, the number of follicles of different sizes (>10, 5-9 and 1-4 mm) between the two groups remained comparable (Table 3). Moreover, the size (mm) of dominant and subordinate follicles (I and II) showed non-significant difference between the groups. Significant different in size of the dominant follicle was observed with time ($p = 0.0466$) (Table 4). With respect to estrus induction using clomiphene citrate, we observed estrus signs in treated animals, but ovulation was not observed in any treated buffaloes in both groups. In contrast, use of clomiphene citrate along with hCG (Human Chorionic gonadotrophin) resulted in ovulation in Sahiwal heifers (Rehman *et al.* 2014, Bukhari *et al.* 2016, Nagendra *et al.* 2022). Also, the follicular profile of small and medium sized follicles was comparable between the clomiphene citrate treated and control animals (El-Sherry *et al.* 2011). Studies in buffaloes have shown that lesser percentage of animals exhibiting estrus following clomiphene citrate (Wankar *et al.* 2017). This variation from previous reports might be difference in the body condition of animals, heat detection methods, season and follicular profile. Though, several researchers consistently reported the efficacy of clomiphene in induction of estrus in cattle and buffaloes at the dose of 300 mg/animal orally (Kankal *et al.* 2008), this study demonstrated that clomiphene citrate enhance the estrus signs without ovulation which supports the use of hCG along with

clomiphene citrate (Rehman *et al.* 2014). The results of our study showed that clomiphene citrate treatment increased the large follicular growth in treated heifers with no ovulation. In other studies, it has been demonstrated that heifers manifested short luteal phase following ovulated (Evans *et al.* 1994). Further studies can be tried with clomiphene citrate along with other hormones to induce the ovulation in buffaloes.

In conclusion, this study shows that body weight, external genital measurements and antral follicular count are correlated and oral administration of clomiphene citrate failed to induce fertile estrus in pre-pubertal Murrah buffalo heifers.

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