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Subclinical Mastitis Affect Ovarian Dynamics, Conception Rate, Early Embryonic Losses, Steroidal Hormones Profile and Genes Expression in Nili Ravi Buffalos

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MA and MA conceived, designed, analyzed and wrote the manuscript. MA and AM executed the study and statistically analyzed the data. MA and AS performed the hormone analysis. FA, SHZ, AU and all authors critically reviewed the manuscript.

Key words
Subclinical mastitis, Nili Ravi Buffalos, Ovsynch, Hormonal profile, Ovarian dynamics, Genes expression

ABSTRACT

The aim of this study was to investigate the effect of subclinical-mastitis on production and reproduction parameters, and genes expression in Nili-Ravi buffaloes. The subclinical mastitis was diagnosed through somatic cells count (SCC) in all selected animals. Study divided into two phases, 1st phase: Ovsynch protocol was applied on subclinical-mastitis (SCM: n=67) and healthy (HAL: n=84) Nili-Ravi buffaloes to investigate hormonal profile, follicular dynamics, conception rate, and embryo losses. However, in 2nd phase (n=12 buffaloes/group), the genes expression pattern was determined through RTPCR. The results showed significantly higher diameter of an ovulatory follicle in HAL buffaloes than SCM (18.1±1.06 vs 15.24±1.49 mm; P≤0.02). There was a significant difference (P≤0.001 and P≤0.002, respectively) in days open and services/ conception between SCM and HAL buffalos. The conception rate at days 30 and 45 after AI was higher (79.8 and 69.1%, respectively; P≤0.04) in HAL buffalos with lower (P≤0.002) embryonic loss. The mean estradiol concentrations were significantly higher (P≤0.04) in HAL buffaloes as compared to SCM on days 8 and 9 of protocol (33.99±0.64 and 42.99±0.65 pg/ml, respectively). However, the serum progesterone concentration was significantly lower (P≤0.04) on post-AI (day 15 and 30 after AI) in SCM buffaloes as compared to HAL. Moreover, in HAL buffaloes, positive correlations (P≤0.02) were observed between hormones, ovulatory follicles and conception rate. There was higher expression of FSHR, GDF9 and BMP15 in HAL buffaloes. Finally, in conclusion, SCM disrupts the productive and reproductive performance, conception rate, hormonal profile, and gene expression in Nili Ravi buffaloes.

INTRODUCTION

Buffaloes are one of the leading dairy animals in developing countries, contributing 12% of total world milk. According to the FAO report, 95% of the world buffalo's population is present in Asian countries, like Pakistan, India, Nepal, Bangladesh, etc. (Mansour *et al*., 2016). However, these animals exhibit a somewhat sluggish frequency of reproduction due to low fertility (Michael *et al*., 2020), inactive ovaries, long calving interval (Mansour *et al*., 2017), and sensitivity to diseases like an introduction to endotoxin (lipopolysaccharide (LPS), exotoxin (*S. aureus* exosecretions) and intramammary infection (Mastitis) (Jinagal *et al*., 2023). In short, buffalos are very sensitive to bacterial toxins and mastitis occurrence. Therefore, heave economic losses in production and reproduction due to mastitis toxins in buffalos.

The occurrence of mastitis among the buffalo population results in lower milk production, increases in the cost of treatment and culling (Hertl *et al*., 2014). The economic impact of clinical mastitis on reproduction failure in dairy animals has increased odds of abortion (Dolecheck *et al*., 2019), abnormal length of inter-service intervals, and failure to become pregnant after natural service or AI due to poor ovarian dynamics (Nava-Trujillo *et al*., 2010).

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Subclinical mastitis is more threatening than clinical mastitis in dairy animals due to long-term/chronic infection, which put a negative effect on ovarian dynamics (either follicular growth or luteal function) (Mansour *et al*., 2017), increases odds of early embryonic losses (Dalanezi *et al*., 2020), low conception rate, and also an imbalance in hormonal profile (Furman *et al*., 2014). Subclinical mastitis increases the secretion of cytokines and other inflammatory mediators. Which depress estradiol and LH concentration (Lavon *et al*., 2010), resulting in delayed ovulation or decreased ovulation rate (Roth and Wolfenson, 2016), disturbed luteal function, and reduced plasma progesterone concentration (Smulski *et al*., 2020). However, to the best of my knowledge, there is no study reported to use Ovsynch protocol in long-term subclinical mastitic buffaloes to investigate their reproduction and genes expression. So, the aim of this study was to investigate (a) the ovarian dynamics, (b) estradiol (E2) concentration and their correlation with ovulatory follicles, (c) progesterone (P4) concentration, (d) impact of SCM on days open and services per conception, (e) conception rate and early embryonic losses and (f) genes expression in long term mastitic Nili Ravi buffalos.

MATERIALS AND METHODS

Animals and management

The study was conducted in three different private dairy farms in district D.I. Khan and Okara-Pakistan. All selected buffalos were fed green fodder and a sufficient quantity of concentrate daily, fresh drinking water was available. The diagnosis of subclinical mastitis through the somatic cells count (SCC) was regularly performed twice a month in all these animals (Hogan *et al*., 1999). The study was approved by the Animal Ethical Committee University of Veterinary and Animal Sciences, Lahore-Pakistan.

Study design and synchronization

All buffalos were clinically examined through ultrasonography (Honda-1600, Japan) for any abnormalities (metritis and endometritis) and confirmation of cyclicity (presence of CL on ovaries). The study was divided into two phases.

(i) 1st phase of study: All selected animals (HAL: n=84 and SCM: n=67) were synchronized by Ovsynch protocol as previously described by (Pursley *et al*., 1995). The Ovsynch protocol consisted of administration of GnRH analogue (50µg Dalmaralin®, Lecirelin acetate, Fatrolecrina Uruguay Ltd, Uruguay) were administered (i.m) on day 0. Then on day 7, PGF2α analogue (150 µg Dalmazin®, Cloprostenol sodium, Fatrolecrina Uruguay Ltd, Uruguay) was administered (i.m) and 48 h apart, GnRH analogue (50 µg Dalmaralin®, Lecirelin acetate, Fatrolecrina Uruguay Ltd, Uruguay) were administered (i.m) at fixed time (16 h apart of second GnRH injection) double AI was performed.

(ii) 2nd phase of study: All selected animals of this phase of study (HAL: n=12 and SCM: n=12) were synchronized by Ovsynch protocol and the buffaloes were slaughter after the PGF2α injection on day 8 and both ovaries were collected. Follicular fluid and ovum was picked from preovulatory follicles and granulosa cells were detached from follicles wall. The follicular fluid, granulosa cells and ova's were preserved at -80 °C until further processing.

Ultrasonography

All buffalos were scanned on days 3, 6, 8, and 9 of protocol for follicular dynamics. Pregnancy diagnosis was performed at 30 and 45 days after first AI. However, those buffalos, which were diagnosed non-pregnant on this scanning, were also confirmed for early embryonic loss (Lavon *et al*., 2010). Days open were recorded for pregnant buffalos from parturition to conception.

Blood sampling and hormone analysis

Blood were collected from jugular vein at pre-protocol (-1 d), day 3, 6, 8, 9 and post AI (day 15 and 30 of AI) and allowed to clot. Serum was separated by centrifugation of 1500×g for 10 min and stored at -20 °C until assay performed.

Serum P4 and E2 concentrations were determined by Radioimmunoassay (RIA) kit (Diagnostic products crop, Los Angeles, CA, USA). The minimum detectable amount for P4 was 0.1 ng/ml, inter and intra-assay coefficient of variation were 6% and 9.9%, respectively, and for E2 was 8 pg/ml and inter and intra-assay coefficient of variation was 6.9% and 11.5%.

Gene expression

A total RNA was isolated from oocytes and granulosa cells by using the GeneJET RNA extraction kit (Thermo scientific) and were transcribed into cDNA by using commercial cDNA Synthesis Kit (Thermo scientific) according to the manufacturer's instructions.

The primers for target genes were designed on Primer-BLAST (NCBI) and listed in Table I. The expression of the target genes in the oocytes and granulosa cells were quantified by using RTqPCR (Rotorgene-Q, QIAGEN). The PCR recipe was 20 µl and initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 sec followed by annealing and extension for 1 min. The relative expression level was analyzed by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table I. Primers for genes expression.

Gene	Primer sequence	Product size (bp)
GDF 9	F:5'-TTTCCCCAGAATGAATGTGAG-3' R:5'-GGCTCCTCCTTACACAACACA-3'	496
BMP15	F:5'-CTCGGATCTTAGGGCATCC-3' R:5'-GATTACTTTCAGGCCCGTCA-3'	696
GAPDH	F:5'-AAGGTCGGAGTGAACGGATT-3' R:5'-TCACGCCCATCACAAACAT-3'	350
FSHR	F:5'- TGGCAAGTGCTTAATACCTGT-3' R:5'- GCAAACGTGTTCTCCAACC-3'	149

GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FSHR, follicle stimulating hormone receptor.

Statistical analysis

The data of variables are presented as mean± SEM, data were analyzed by using statistical software SPSS-22 version. Normal distribution of serum E2 and P4 was assessed by Shapiro wilk test. Students t-test was performed to assessed size of small, medium and ovulatory follicle, no,s of AI (services), days open, days of first AI (service) and to compared serum P4 and E2 concentrations among groups. Effect of SCM on conception rate and embryonic losses were determined by chi square. The Pearson correlation coefficient was measure between (a) ovulatory follicle and serum E2, (b) serum P4 and conception rate. The relative genes expression data was analyzed by student t-test. The level of statistical significance for all variables was $P<0.05$.

RESULTS

Milk yield at lactation period

Effect of mastitis on milk yield in Nili-Ravi buffalos is presented in Table II. There was significant difference ($P<0.04$) in monthly milk yield between SCM and HAL group. The average total milk yield in SCM vs HAL was (892.54±15.45 vs 2055.58±12.56 kg, respectively).

Reproductive parameters

The mean size of small follicles and ovulatory follicles was significantly higher ($P< 0.04$) in HAL vs SCM (5.7±1.29 and 18.1±1.06 vs 3.5±0.65 and 15.2±1.49 mm, respectively), however, no significant difference in the mean diameter of medium size follicle (11.17±1.12 vs 8.12±1.29 mm, respectively) (Fig. 1). Moreover, the conception rate at day 30 and 45 of AI was significantly higher ($P<0.03$) in HAL (79.8 and 69%, respectively) as compared to SCM (61.19 and 37.32%, respectively). There were significantly higher ($P<0.002$) embryo losses in SCM as compared to HAL. The days open were significantly higher ($P<0.02$) in SCM vs HAL buffalos (406.5±3.87 vs 191.1±1.73; respectively) and no,s of AI to conceived (services/conception) SCM buffalos were significantly higher ($P<0.04$) as compared to HAL (Table III).

Table II. Effect of long-term subclinical mastitis on milk yield in nilli Ravi buffalos.

Months vice milk yield	Groups	
	SCM	HAL
1st month	211.8±1.79^b	239.98±1.53^a
2nd month	171.21±2.13^b	236.57±1.82^a
3rd month	142.65±1.93^b	231.76±1.64^a
4th month	117.47±2.13^b	221.44±1.81^a
5th month	95.65±2.15^b	210.69±1.83^a
6th month	72.62±2.03^b	204.76±1.73^a
7th month	41.32±1.77^b	197.31±1.51^a
8th month	26.13±1.78^b	186.77±1.52^a
9th month	11.36±1.83^b	172.51±1.56^a
10th month	2.29±2.51^b	153.77±2.14^a
Total milk yield in lactation period	892.54±15.45^b	2055.58±12.56^a

Monthly milk yield and total milk yield of mastitis positive (SCM, n=62) and mastitis negative (HAL, n=84). Milk yield are presented in litter and different superscripts denoted significant level ($P<0.05$). SCM, subclinical mastitis, HAL, healthy.

Table III. Effect of long term subclinical mastitis on reproductive performance in nilli Ravi buffalos.

Reproductive variables	Groups	
	SCM	HAL
Days open (Maen±SEM)	406.5±3.87^b	191.1±1.73^a
No,s of days from calving to 1st AI (Maen±SEM)	128.9±4.38^b	120.5±3.16^b
No,s of AI for conception (Maen±SEM)	3.13±0.12^b	1.56±0.06^a
Conception rate at day 30 of AI	61.19% (41)^b	79.8% (67)^a
Conception rate at day 45 of AI	37.32% (25)^b	69% (58)^a
Embryo losses	39% (16/41)^b	13.4% (9/67)^a

Different reproductive variables of Mastitis positive (SCM, n=67) and mastitis negative (HAL, n=84). Pregnancy diagnosis through ultrasound 30 and 45 days after AI, those which conceived at day 30 and observed non-pregnant at day 45 after AI, confirmed early embryonic loss. Different superscripts denoted significance level ($P<0.05$).

Steroid hormones profile

The mean basal level of serum E2 concentrations were significantly lower ($P< 0.0001$) in SCM as compared to HAL before the starting of protocol (11.14±0.31 pg/

ml vs 15.80±0.55, respectively). Moreover, mean E2 concentrations were significantly increasing in both groups during experimental protocol. However, the highest concentration was at day 8 and 9 of protocol in HAL as compared to SCM buffalos (33.99±0.64 and 42.99±0.65 vs 23.44±0.67 and 31.86±0.74 pg/ml, respectively; P<0.0001) as (Fig. 2A). However, there was no significant difference between SCM and HAL buffalos in serum P4 concentration before starting of protocol. Moreover, overall mean concentration of serum P4 was significantly lower (P< 0.03) in SCM as compared to HAL buffalos. The highest concentration was found at the time of PG injection in SCM and HAL buffalos (27.74±1.45 vs 44.29±1.12, respectively; P<0.0001) and post AI as (Fig. 2B).

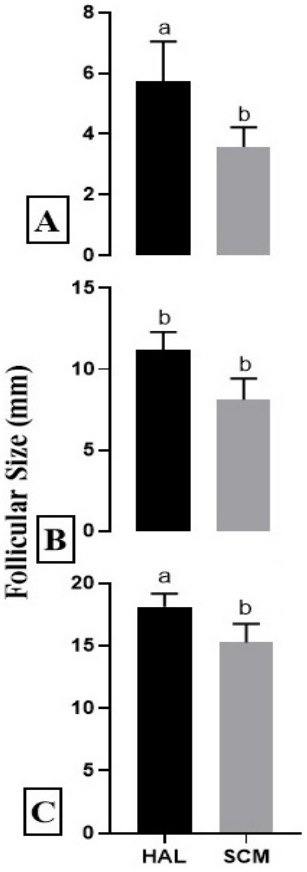

Fig. 1. Characterization of follicular growth, x-axis shows healthy buffalos (HAL) and subclinical mastitis (SCM), while Y-axis shows size of follicles in mm. In HAL, small size follicles (3.5-8.5), medium size follicles (9-14) and ovulatory follicle (15-21), while in SCM, small size follicles (2.5-5), medium size follicles (6-13) and ovulatory follicles (13-18). Different letters shows significance difference (P<0.05) between groups.

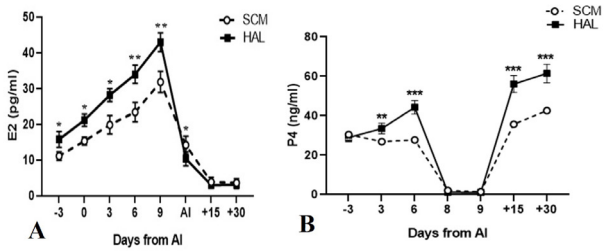

Fig. 2. (A) Serum estradiol concentration (pg/ml) in pregnant subclinical mastitis (SCM) and healthy (HAL) buffalos at -3, 0, 3, 6, 8, 9 and post AI of protocol (+15 and +30). (B) Serum progesterone concentration (ng/ml) in pregnant subclinical mastitis (SCM) and healthy (HAL) at pre AI (pre-protocol, day 3, 6, 8 and 9) and at post AI (day 15 and 30). Results are expressed as mean±SEM, Asterisk shows significant difference between groups (P<0.05).

Table IV. Pearson correlation between serum estradiol concentration and ovulatory follicles, and between serum progesterone concentration and conception rate.

	HAL		SCM	
	r value	**P value**	**r value**	**P value**
Serum E2 concentration with ovulatory follicles				
Day 8	0.79	0.02	0.68	0.05
Day 9	0.83	0.03	0.72	0.04
Serum P4 concentration with conception rate				
Day 15	0.71	0.01	0.41	0.1
Day 30	0.86	0.02	0.24	0.3

Mastitis positive (SCM, n=17) and Mastitis negative (HAL, n=17). Pearson correlation "r" and degree of significance "P". The Serum estradiol "E2" and serum progesterone "P4".

Table IV shows that the positive correlations were observed between conception rate and post AI P4 concentration in HAL buffalos (r=71 and 0.86; P<0.01 and 0.02, respectively), however no relationship was found in SCM buffalos. Moreover, positive correlations were observed between ovulatory follicles and E2 concentration at day 8 and 9 in both groups.

Genes expression level in oocytes and granulosa cells

The level of mRNA expression of GDF9 in the oocytes were significantly (P=0.01) lowered in the SCM buffaloes as compared to HAL, as shown in Figure 3. Similarly, the expression of genes associated with folliculogenesis (BMP15) in the oocytes were significantly (P=0.03) lowered in the SCM buffaloes as compared to HAL (Fig. 3). This indicated that SCM affected the oocytes quality and folliculogenesis. Furthermore, the level of FSHR in

the granulosa cells were significantly (P=0.01) in the SCM buffaloes as compared to HAL (Fig. 3).

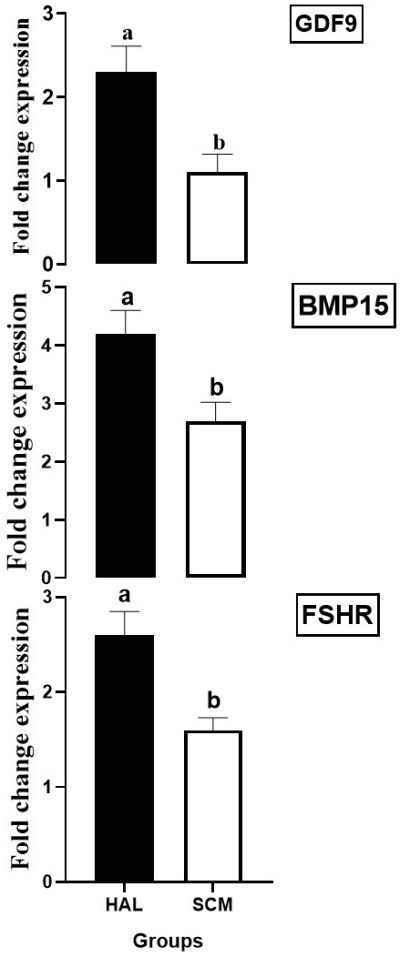

Fig. 3. Expression of mRNA for GDF9, BMP15 and FSHR genes in oocytes and ganulosa cells collected from preovulatory follicles (phase 2). RNA was extracted from Healthy "HAL" (n=12) and Subclinical Mastitis "SCM" (n=12) buffaloses. Data are presented as mean±SEM. Different letters denoted significant difference at P<0.05. The primers for target genes were designed on Primer-BLAST (NCBI) and listed in Table I.

DISCUSSION

Multiple reports concluded that intramammary infection (IMI), either clinical or subclinical caused a deleterious impact on the reproductive performance of dairy animals (Roth and Wolfenson, 2016; Edelhoff *et al*., 2020). To the best of our knowledge, this is the first study on the impact of long-term subclinical mastitis on reproductive performance and genes expression in buffalos. The main finding of this study indicates that long-term subclinical mastitis exhibits a deleterious effect on the reproduction of buffalos.

The result of the current study showed, the SCM put a deleterious impact on milk yield in SCM buffalos as compared to HAL and decreases day by day in buffalos, as pervious reported in cattle (Holstein Frisian) (Salvador *et al*., 2012; Gupta *et al*., 2015). The main reason for the low production of milk yield might be due to heavy bacterial load (Lavon *et al*., 2011), which produce Exo and endotoxin and increase the number of leukocytes in the udder (Jinagal *et al*., 2023). The second possible reason for low milk yield in mastitic dairy animals has increased body temperature, red warm and swollen mammary quarters (Hertl *et al*., 2014). SCM also affects the reproductive performance in dairy cattle and ewes (Roth *et al*., 2013).

The result of the current study; SCM cannot effect on days of 1st insemination (service). It may be due to the normal involuntary period and normal first estrous cyclic. However, in previous reports, clinical mastitis and subclinical mastitis in dairy animals just after parturition increases days to first service (Mansour *et al*., 2016). The possible reason is that increasing the number of inflammatory mediators (cytokines, tumor necrosis factors, interleukins, and interferon), which directly affects the reproductive system of dairy animals (Mansour *et al*., 2017; Koh *et al*., 2018). Moreover, in the current study, services per conception (no's of insemination/conception) and days open were greatly increased in SCM as compared to HAL buffalos. These findings were in corroboration of other studies (Hertl *et al*., 2014). The possible mechanism of this deleterious effect of long term SCM in buffalos is the release of inflammatory mediators (cytokines, TNFα and IL-1β) (Dolecheck *et al*., 2019), which causes a deleterious impact on steroid hormone profile (estradiol), GnRH secretions and delay or block LH pulse (Roth and Wolfenson, 2016). Besides this, long term SCM affect the ovarian dynamics (follicular growth and luteal growth) and also delays the oocytes maturation and fertilization process, because oocytes maturation (expansion of COC) is governed by LH hormone, which provides glucose for nourishment and pyruvate for oxidation to oocytes (Hertl *et al*., 2014; Edelhoff *et al*., 2020). However, SCM increases the nitric oxide (NO) in milk, which resulted in to increase in the PGF2α concentration in milk and blood causing premature luteolysis (Smulski *et al*., 2020). Another possibility is that increase of SCM reduces the expression of growth differentiation factor-9 (GDF-9) and octamer-bending transcription factor-4 (OCT-4) genes, which are oocytes maturation and early embryonic development factors (Roth *et al*., 2013; Saleem *et al*., 2023).

Moreover, many researchers documented that CM or SCM disrupts ovarian dynamics; either follicular or CL

growth, and their respective functions (Lavon *et al*., 2010). In the present study, the SCM significantly decreased the size of the ovulatory follicle, the conception rate at day 30 or 45, and increased embryonic mortality. However, it was previously reported that the conception rate was too low (Roth and Wolfenson, 2016; Mansour *et al*., 2017). The possible reason for the difference in conception rate between the previous report may be synchronization protocol (Pursley *et al*., 1995). The early embryonic losses may be due to the poor autotrophic effect which is induced by abnormally increased secretion of PGF2α in the bovine uterus. Moreover, it is reported that heat stress and SCM put a negative impact on oocytes competency, fertilization process, and blastocysts formation in bovines (Smulski *et al*., 2020; Saleem *et al*., 2023). Besides this one possible reason is that increased secretion of cortisol blocked the secretion of LH, which delayed ovulation or oocytes competency (Roth *et al*., 2013; Dalanezi *et al*., 2020). Interleukin-6 block the secretion of estradiol, whereas tumor necrosis factor β and interferon δ insert toxic effect on CL and reduction in P4 concentration (Koh *et al*., 2018). Moreover, tumor necrosis factor α increase the blastomeres in the embryo at day 5, which was apoptosis and decrease the inner cell mass and lead to decreased survival of embryos (Jinagal *et al*., 2023).

In current study the hormonal profile disruption is reported in SCM compared to HAL buffalos, which is similar to various documented reports (Furman *et al*., 2014; Mansour *et al*., 2017). This deleterious effect of SCM on hormonal profile may be due to reduced expression of LHCGR, steroid genes in theca or granulosa cells (Hertl *et al*., 2014; Mansour *et al*., 2016). The one possible reason of estradiol depression is due to reduced expression of CYP19AI and CYP11AI. Whereas, lower P4 concentration in SCM could be due to CL size and interference in its developmental process (Edelhoff *et al*., 2020). Data from the current study showed that low level of steroid hormones, low pregnancy rate and poor folliculogenesis in SCM buffalos result from the abnormally low level of expression of genes in oocytes and granulosa. The reduction in the level of FSHR and BMP15 mRNA may directly involve in the reduced secretion of estradiol and FSH, which lead to low growth of follicles on ovaries (Saleem *et al*., 2023). The possible mechanism of this deleterious effect of SCM in buffalos is the release of inflammatory mediators (cytokines, TNFα and IL-1β) (Koh *et al*., 2018; Dalanezi *et al*., 2020).

CONCLUSION

In conclusion, SCM disrupts the milk yield, follicular growth, and conception rate on days 30 and 45. There was appeared to be due to lower estradiol and progesterone concentration which also increased early embryonic mortality in SCM buffalos. The days open and services per conception also increased in SCM buffalos as compared to HAL buffalos. Furthermore, the genes expression lowers in SCM buffaloes, which indicate lower cleavage and blastocysts rate than HAL. It means chronic or long-term subclinical mastitis threatens productive and reproductive performance in buffalos.

DECLARATIONS

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IRB approval

This work was approved by the Board of Studies, UVAS, Lahore, Pakistan (Reference No. 1016/2019).

Ethical statement

All procedures of present study were performed according to animal protocols approved by the Institutional Animal Care Committee standards and strict follow rules and regulation of University Ethical Committee.

Generative AI and AI-assisted technology statement

The authors declare that no genrative AI was used in the creation of this manuscript.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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