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Effect of mastitis on luteal function and pregnancy rates in buffaloes

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ABSTRACT

The aim of this study was to investigate the effects of mastitis on CL development and function and pregnancy rate in buffaloes. Sixty-six buffaloes (*Bubalus bubalus*) reared in a commercial farm at El-Beheira governorate, north of Egypt were used in this study. According to the visual observation of milk, physical examination of the udder and actual somatic cell count in milk, buffalo cows were divided into three groups: without mastitis (W), n = 23; subclinical mastitis (SC), n = 18; and clinical mastitis (C), n = 25. All buffalo cows were synchronized by double dose of $PGF2_{\alpha}$ (11-day interval) and inseminated by frozen-thawed semen of fertile bull. Mean CL diameter was ultrasonically examined on Days 5, 9, 12, 16, 21, and 25 after artificial insemination (AI). Blood samples were taken on the days of ultrasonography for progesterone (P4) assay. Results indicated that pregnancy rates were lower ($P < 0.05$) in C (28.00%) and SC (55.56%) compared with W (69.57%) on Day 25 after first AI. Pregnancy rates reduced to 60.87%, 44.45%, and 16.00% in W, SC, and C, respectively, at Day 45 after insemination. Thus, the embryonic loss was 8.7%, 11.11%, and 12.00 % in W, SC, and C cows, respectively. Pregnancy rates decreased between 44.32% and 50.51% when mastitis occurred during Day −15 before to Day +30 after AI, compared with 59.22% in the uninfected cows. The diameter of CL was greater ($P < 0.05$) in W than SC and C cows starting at Day 9 postbreeding onward. Likewise, P4 concentrations on Days 9 through 25 after AI were greater ($P < 0.05$) in W cows as compared to SC and C cows. Positive correlations ($P < 0.01$) were found on Days 5, 9, 12, 16, 21, and 25 after AI between CL diameter and P4 concentrations. Similar trend was found among CL diameter, P4 concentrations, and pregnancy rate. Accordingly, incidence of mastitis revealed suppression to both CL diameter and function leading to significant reduction in pregnancy outcome of buffalo cows.

1. Introduction

Buffaloes are recognized to have economic significance among livestock animals in terms of milk and meat yields as well as work purposes [1]. Mastitis is an infection of the mammary gland, which is usually correlated with physical, chemical, and bacteriological changes in the milk and pathologic changes in the glandular tissue of the udder [2]. Bovine mastitis is an important and a persistent infection in the buffalo population culminating in economic losses; drop in milk production, increases in the cost of treatment, and culling process [3,4]. Buffaloes have some characteristics that may contribute to greater risk of mastitis. For example, the udder is more pendulous, and teats are longer in comparison with cattle [5]. Somatic cells count (SCC) is an indicator of both resistance and susceptibility of animals to mastitis and can be used to monitor the level or occurrence of subclinical mastitis in herds or

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individual cows [6]. The SCC also has been used for buffaloes in mastitis diagnosis, and in fact, it seems probable that a SCC greater than 200,000 cells/mL is an indicator of an udder infection [7,8]. Clinical mastitis is manifested by secretion of abnormal milk (i.e., watery milk, presence of flakes in milk, and so forth) and/or inflammation (i.e., redness, swelling, hardness, and so forth) of the mammary gland [9]. Subclinical mastitis (i.e., the asymptomatic inflammation of mammary tissue) is the most common form of mastitis representing 15 to 40 times higher incidence than clinical cases [10]. Subclinical mastitis is a bigger concern than clinical mastitis because it remains undetected without the use of SCC, a measure not available to regular raisers of dairy animals [11].

Luteal size and progesterone (P4) secretion is an important indicator for functional CL in buffaloes [12,13]. The development and function of the CL differs between pregnant and nonpregnant buffalo cows [13]. The rate of CL growth between Days 5 and 10 after artificial insemination (AI) could be a more accurate indicator of CL function, and a predictor of the likelihood of pregnancy in buffalo cows [14]. Also considered important was the increase in CL area between Days 15 and 20 after breeding in pregnant buffalo cows and the decrease in CL area in nonpregnant buffalo cows during same period [13]. The secretion of P4 during early luteal phase is essential for successful establishment of pregnancy [15]. Low plasma P4 concentration during early luteal phase was shown in nonpregnant buffalo cows compared with their pregnant counterparts [16]. Studies in dairy cattle have revealed associations between clinical mastitis and increased odds of abortion [17], abnormal length interservice intervals [18], and failure to become pregnant after a service [19]. Other studies have identified associations between subclinical mastitis as measured by increased individual-cow SCC and increased odds of embryonic loss [20], abortion [21], and failure to become pregnant to first service [21]. Several potential mechanisms have been proposed to explain the effect of mastitis on reproductive performance. These are comprehensively reviewed by Hansen et al. [22], but broadly encompass detrimental impact of inflammatory mediators on ovarian follicular function [23], intrauterine embryonic survival [24], decreased luteal-phase length [18], and the balance of luteolytic versus luteotrophic prostaglandins after conception [25,26]. Besides, possible reason that mastitis has an inhibition effect on gonadotropin secretion leading to reduced gonadotropin support for ovulation, oocyte maturation, folliculogenesis, and luteal function [22]. Mastitis is associated with increased secretion of cytokines that in turn can inhibit secretion of LH and reduces circulating concentrations of P4 [22]. However, virtually all the published information about the risk factors for mastitis refers to dairy breeds of cattle but little information is available for buffaloes. Although a high probability exists that these identified risk factors may also be observed among these species. Therefore, the objectives of this study were to evaluate the effects of mastitis on development and functions of the CL and consequently, its effect on pregnancy rates in buffaloes. It was hypothesized that both clinical and subclinical mastitis might be associated with a reduction in CL functionality leading to pregnancy reduction in buffaloes.

2. Materials and methods

2.1. Animals and management

Sixty-six lactating and cyclic buffalo cows (*Bubalus bubalus*) reared in a commercial farm at El-Beheira governorate, north of Egypt, where 50% of the yard area was sheltered were chosen for this study. Cows were at postpartum (average 181 ± 21.65 days) at the commencement of the experiment. Average body condition score (BCS) of cows according to Lowman et al. [27] was 3.3 ± 0.5 (range: 2.5–4). Cows were fed according to the recommendation of the National Research Council standards for buffaloes [28,29]. Briefly, buffaloes were fed on green fodder (*Trifolium alexandrinum*) with an adequate amount of concentrate mixture (maize or wheat 60%, soybean 25%, wheat bran 10%, rice bran 5%, and common salt 1%). Mineral-balanced mixture blocks and clean drinking water were offered as free choice. Cows were hand milked twice daily, and the average milk yield was 12.65 ± 2.87 kg/day.

2.2. Experimental design

Consistent with the National Mastitis Council recommendation [30] and according to the visual observation of milk, physical examination of the udder and actual SCC in milk, the buffalo cows were randomly divided into three groups. First group cows (without mastitis [W], n = 23) in which milk has a white appearance and free of flakes, clots, and other gross alterations in appearance and has SCC less than 200,000/mL before or after first AI. Second group (subclinical mastitis [SC], n = 18) cows had no clinical changes in milk; however, SCC exceeds 200,000 cells/mL before or after first AI (SCC elevated between 200,000–600,000 cells/mL). Third group (clinical mastitis [C], n = 25) cows had abnormal milk with flakes, clots, and other gross alterations plus physical inflammation of the udder (i.e., swelling, hardness, and so forth) and SCC greater than 600,000/mL before or after first AI. Each of the previously mentioned three groups was further divided into four subgroups of 15 days each according to the time of mastitis diagnosed before first AI: −30 to −16 and −15 to −1 days and after first AI: +1 to +15 and +16 to +30 days. Data from Days −1 to +1 around detected estrus and AI were not shown.

2.3. Estrus synchronization and AI

All buffalo cows were synchronized by the double dose $PGF_{2\alpha}$ at 11 days apart protocol (500 μg intramuscular, Estroplan, Cloprostenol sodium; Parnell Laboratories New Zealand Ltd., New Zealand). Before applying the synchronization protocol, an ultrasound examination was applied on all cows to identify the cyclic and noncyclic ones, on the basis of presence of a CL [31]. Based on the ovarian cyclicity, 66 cows were cyclic. After 72 hours of the second $PGF_{2\alpha}$ injection, signs of induced estrus were monitored by visual observation, teaser bull, and transrectal palpation. Based on the estrus observation, all cows were detected in estrus and had one opportunity to be AI. First AI was carried out during mid-to-late estrus, and subsequent inseminations

were carried out at 24-hours interval. Artificial insemination was done using frozen-thawed semen from a bull of known high fertility by the same operator.

2.4. Milk sample collection

After complete evacuation of the milk, visual observation of milk and physical examination of the udder was conducted. The relevant data including lactation number, date of calving, and age were recorded for each cow. Milk samples were collected twice per day at early morning (6 AM) and evening (5 PM). Duration of daily milk samples collection was 1 month before and 1 month after AI. Milk samples were collected aseptically after cleaning of the teats with 70% alcohol and leaving the first few striping's drops of milk. Samples were transported to the laboratory in cool containers until SCC tests were performed.

2.5. Somatic cell analysis

A total of 528 quarters were examined for the presence of mastitis by testing the SCC. The SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley 150, Bentley Instruments, Inc., USA). Ethidium bromide dye was used for specific binding to DNA in cell nuclei. By using a process known as pulse height analysis, the pulses are sorted, counted, and translated into an SCC.

2.6. Ultrasound examination

All buffalo cows were examined by the same operator using a real time B-mode ultrasound scanner (Falco Vet, Esaote/Piemedical, Maastricht, Netherlands) equipped with a 6 to 8 MHz linear-array rectal transducer. The scanner was supplied with a video-graphic printer (Sony UP-895, Sony Corporation, Japan). The diameter of CL was examined on Days 5, 9, 12, 16, 21, and 25 after AI. Once the ovary was visualized, the image was adjusted to give a better definition of the CL and then frozen for measuring the long and short axis (diameter of the CL = short axis + long axis/2) [13]. Twenty-five days after first AI, cows underwent transrectal ultrasonography to assess embryonic development by visualizing the presence of the embryo and a proper heartbeat rate. Later pregnancies were confirmed on Day 45 after AI by transrectal ultrasonography. Cows with embryonic development on Day 25 but nonpregnant on Day 45 were considered to have undergone late embryonic mortality [13].

2.7. Blood sampling and P4 RIA

Blood samples were withdrawn from the jugular vein of each animal immediately after ultrasound scanning (Days 5, 9, 12, 16, 21, and 25 after AI). Blood samples were centrifuged for 15 minutes at 1200 × *g* within 2 hours after collection. Sera were harvested and stored at −20 °C until P4 was determined. Serum P4 levels were measured by RIA according to El-Banna and Gamal [32]. The accuracy of the assay was tested by two standard curves using buffer matrix and were superimposed indicating a high degree of accuracy at all concentrations of P4. Serum P4 concentration values greater than 1.5 ng/mL were considered indicative of the presence of an active CL [33]. The minimum detectable amount of P4 was 2.1 ± 0.1 pg, and the intra-assay and interassay coefficients of variation were 6.2% and 11.8%, respectively.

2.8. Statistical analysis

Milk yield, parity, days postpartum, and BCS were found not significant between groups and were excluded from the final statistical model. Data were analyzed using the two-way analysis of variances by using the following model.

$$Y_{ijk} = \mu + G_i + T_j + G_iT_j + e_{ijk}$$

Where: Y_{ijk} = an observation taken on the kth buffalo cows;

μ = overall mean;

G_i = a fixed effect of the ith group of mastitis;

T_j = a fixed effect of the jth time of mastitis diagnosed before and after first AI; and

e_{ijk} = Random error assumed to be independent and normally distributed; with mean = 0 and variance = σ2e.

Data for CL diameter, pregnancy rates, and embryonic mortality among different groups were analyzed by general linear model-least square analysis of variance. Concentrations of P4 were measured by the repeated measure ANOVA. Correlation analyses were performed among SCC, CL diameter, P4 concentrations, and pregnancy proportion. All statistical analyses were performed using Predictive Analytics SoftWare (PASW) statistic 18.0 (SPSS, Inc., Chicago, IL, USA) [34].

3. Results

3.1. Pregnancy rates and embryonic losses

Buffalo cows in all groups were almost similar in parity (mean = 3.75 ± 0.62), BCS (mean = 3.25 ± 0.49), days postpartum (mean = 181 ± 21.65 days), and average milk yield (mean = 12.65 ± 2.87 kg). A high proportion (66/66; 100%) of synchronized buffaloes was confirmed in estrous and inseminated. First-AI pregnancy rates and embryonic losses are reported in Table 1. Overall pregnancy rate on Day 25 after first AI was 50.00%. However, 39.39% of buffalo cows were pregnant on Day 45 after AI revealing a late

Table 1
Pregnancy rates and embryonic losses (%) in control (W), subclinical (SC), and clinical (C) mastitis-buffalo cows on Days 25 and 45 after first artificial insemination.

Group	Pregnancy rate (%)		Embryonic losses rate (%)
	Day 25	Day 45	
W (n = 23)	(69.57)[a]	(60.87)[a]	(8.70)[a]
SC (n = 18)	(55.56)[b]	(44.45)[b]	(11.11)[b]
C (n = 25)	(28.00)[c]	(16.00)[c]	(12.00)[b]

[a,b,c]Means in the same column with different superscript significantly differ at ($P < 0.05$).

embryonic loss of 10.61%. Pregnancy rate on Day 25 and Day 45 after AI in control (W) was higher ($P < 0.05$) compared with subclinical (SC) and clinical (C) buffalo cows. Likewise, embryonic losses were higher ($P < 0.05$) in buffalo cows with clinical and subclinical mastitis compared with control (Table 1). Also, overall first AI-pregnancy rates were lower ($P < 0.05$) in infected (SC and C) cows during Day −15 before to +30 after AI, compared with uninfected (W) cows (Fig. 1).

3.2. Corpus luteum development and P4 concentration

The CL diameter in all buffalo cows is illustrated in Figure 2A. The CL diameter in C cows was smaller ($P < 0.05$) on Day 9 after AI as compared to SC and W cows (13.76, 14.09, and 14.22 mm for C, SC, and W cows, respectively). Similar trend was found thereafter during Days 12 to 25 after AI.

Concentrations of P4 in buffalo cows are shown in Figure 2B. On Day 5 after AI, blood P4 concentrations were not significant ($P > 0.05$) among groups. However, P4 concentrations were higher ($P < 0.05$) on Day 9 after AI in W (2.12 ng/mL) compared with SC (1.79 ng/mL) and C (1.73 ng/mL) cows. Likewise, P4 concentrations on Days 12 through 25 after AI were greater ($P < 0.05$) in W compared to SC and C cows. Percentage of decrease of peripheral P4 ranged from about 15% to 50% in SC and C compared with W cows.

3.3. Correlations

Positive correlations ($P < 0.01$) were found on Days 5, 9, 12, 16, 21, and 25 after AI between CL diameter and P4 concentrations ($r = 0.645$, 0.716, 0.720, 0.663, 0.676, and 0.645 for Days 5, 9, 12, 16, 21, and 25, respectively). Also, positive correlations ($P < 0.01$) were found between pregnancy rate and CL diameter ($r = 0.737$, 0.956, 0.953, 0.958, 0.956, and 0.958 for Days 5, 9, 12, 16, 21, and 25, respectively). Besides, positive correlations ($P < 0.01$) were found between pregnancy rate and P4 concentrations ($r = 0.737$, 0.817, 0.822, 0.757, 0.772, and 0.737 for Days 5, 9, 12, 16, 21, and 25, respectively).

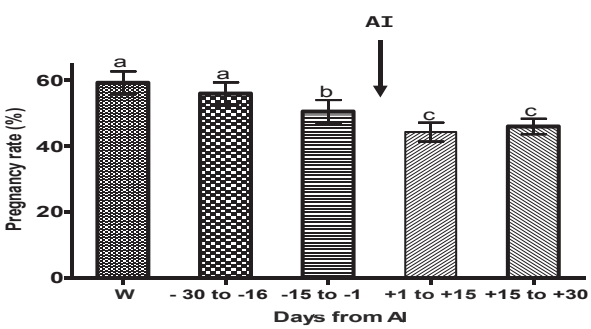

Fig. 1. Pregnancy rate (%) in control (W, n = 23) and mastitis-buffalo cows (subclinical [SC, n = 18] and clinical [C, n = 25]) during 15-day periods before or after AI. Data are presented as mean ± standard error of the mean. Different letters denote significant differences at $P < 0.05$. AI, artificial insemination.

4. Discussion

The aim of this study was to evaluate the effects of mastitis on development and functions of the CL and elucidating the effects of mastitis on pregnancy rates in buffaloes. In the present study, a relatively high percent of embryonic mortality is related with the occurrence of a certain degree of mastitis. Also, results indicate that mastitis incidence at Day −15 (before) and Day +15 (after) AI was associated with a significant reduction in probability of pregnancy rates in buffalo cows. Similar findings were demonstrated in dairy cows [35–39]. Cows with clinical or subclinical mastitis at the time of service exhibited a significant reduction in pregnancy rate [39]. Moreover, cows with clinical mastitis in the periods shortly before and shortly after first service have a negative relationship with fertility [35,37]. Also, presence of subclinical mastitis between Days 1 and 30 after service was associated with a large decrease in pregnancy rate [39]. Reduction in pregnancy rate could be related to the release of inflammatory mediators such as PGF2α, which can alter the interestrus interval by causing premature luteolysis [38]. Furthermore, observations of cows with mastitis suggest that an activation of inflammatory or immune responses external to the reproductive tract can lead to embryonic mortality [22]. An invasion of the microorganisms to the mammary gland might lead to the release of lipopolysaccharide, proteoglycans, and other molecules of bacterial origin that perhaps activate inflammatory and immune responses. This results in an increased cytokine synthesis from the mammary gland in the lymph nodes draining in the mammary glands. Certain cytokines are directly inhibitory to oocyte and embryonic function [22]. Low pregnancy with high embryonic mortality rates in buffaloes with clinical or subclinical mastitis in the present study, support that mastitis is related to trouble conceiving in buffaloes, resulting in an economic loss associated with reproductive failure.

In the present study, CL diameters were larger in buffalo cows without mastitis (W) from Day 12 after AI onward as compared with cows having symptoms of clinical (C) or subclinical (SC) mastitis. Likewise, P4 concentrations were higher in buffalo cows without mastitis (W) compared with clinical (C) or subclinical (SC) mastitis-cows. After cow breeding, mastitis may interfere with CL formation and regression, P4 secretion and embryonic development [40]. Mastitis is associated with increased secretion of cytokines that in turn can inhibit secretion of LH and reduces circulating concentrations of P4 [22]. Secretion of LH can also be blocked by cortisol, a hormone whose secretion can be elevated during mastitis [25]. The decrease or lack of LH secretion may result in compromised follicular and oocyte development, lack of ovulation, and suboptimal luteal function. Also, some of the cytokines produced during mastitis also have a direct effect on the ovaries. Interleukin 6, e.g., blocks the secretion of estradiol [41], leading to a reduction of LH secretion, whereas tumor necrosis factor β and interferon δ are cytotoxic to the CL [42] and could cause reduction in concentrations of P4. It has been shown that the expression of vascular endothelial growth factor by the CL changed during the estrous cycle and was related to circulating concentrations of P4 in buffaloes [43]. It could be speculated that

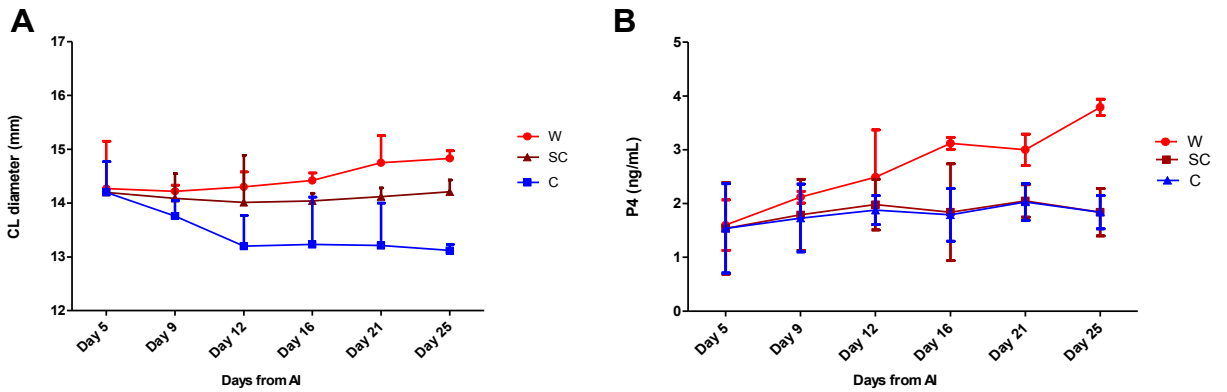

Fig. 2. (A) CL diameter profiles (Mean ± standard error of the mean [SEM]) in control (W, n = 23), subclinical (SC, n = 18), and clinical (C, n = 25) mastitis-buffalo cows on Days 5, 9, 12, 16, 21, and 25 after artificial insemination (AI). (B). Plasma progesterone (P4) profiles (Mean ± SEM) in control (W, n = 23), subclinical (SC, n = 18), and clinical (C, n = 25) mastitis-buffalo cows on Days 5, 9, 12, 16, 21, and 25 after artificial insemination.

the expression of vascular endothelial growth factor by the CL is greater in buffaloes without mastitis compared with buffaloes with clinical or subclinical mastitis. The present study shows that differences in CL diameter between buffaloes without mastitis and these with clinical or subclinical mastitis were associated with differences in concentrations of P4 and pregnancy rate. Progesterone supports the role of the uterus in embryonic development [44], and it is hypothesized that the greater CL diameter accompanied with increased P4 concentrations observed in buffaloes without mastitis enhanced the function of the uterus, and increased the likelihood of pregnancy. Besides, lipopolysaccharide derived from gram-negative bacteria such as *Escherichia coli* causes mammary inflammation and leads to suppression of both structure and function of the CL of diestrus cows [45]. However, it is important to note that the exact mechanism(s) that account for mastitis suppression of fertility in buffalo cows have yet to be resolved.

In conclusion, clinical and subclinical mastitis in buffalo cows affected CL function and lowered pregnancy rate.

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