REPRODUCTIVE CYCLE STAGE BIAS IN PHYSIOLOGICAL AND IMMUNE RESPONSES TO ENDOTOXIN CHALLENGE IN MURRAH BUFFALOES (*Bubalus bubalis)*

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ABSTRACT

Reproductive cycle in large domestic animals has a distinctly different hormonal milieu with estrus dominated by estrogen and diestrous by progesterone. Since the sex steroids are caught up in disease severity, present study investigated variability in immune responses upon endotoxin challenge at day 0 (estrous) and day 10 (diestrous) of the reproductive cycle in Murrah buffaloes. Physiological responses like rectal temperature, heart rate, pulse rate and immune responses like plasma TNFα, nitric oxide, xanthine oxidase, cortisol and glucose were evaluated before and after LPS challenge (*E. coli* 055:B5 0.6 µg/kg body weight). Physiological as well as immune responses were heightened during estrous as compared to the diestrous stage of the cycle after endotoxin challenge. The area under the curve (AUC) for rectal temperature and heart rate was significantly ($P<0.05$) higher at estrous as compared to diestrous. Integrated responses of TNFα, nitric oxide and xanthine oxidaes to LPS challenge calculated as AUC were significantly ($P<0.05$) higher at estrous as compared to the diestrous stage of the estrous cycle. AUC for plasma cortisol, an anti-inflammatory mediator, was significantly ($P<0.001$) higher at the diestrous than the estrous stages of cycle. During first 2 h of endotoxin insult, hyperglycemia was registered which culminated in hypoglycaemia. Total leukocytes were higher in the estrous than the diestrous stage but endotoxin challenge did not affect the total counts. However, there was neutrophilia after endotoxin challenge in both the stages of the reproductive cycle in buffaloes. The results indicate that the underlying physiological attributes of stage of reproductive cycle represents a source of variability in immune competence when challenged.

Keywords: Murrah buffaloes, *Bubalus bubalis*, Reproductive cycle, LPS, TNFα, Nitric oxide, Cortisol

INTRODUCTION

The buffalo is an economically important livestock species in Asian and Mediterranean countries with India having 56% of total world buffalo population which hold good potential for production. Several lines of investigation have reported that sex steroids influence the immune response to the antigenic challenges and outcome of the insult (Olsen and Kovacs 1996; Jorg *et al.*, 1998; Giltay *et al*., 2000; Losonczya *et al*., 2000; Marco *et al*., 2009). Many workers have demonstrated that the hormone environment

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at the time of infection has a profound effect on the outcome of microbial infection in the female reproductive tract (Ramadan *et al.,* 1997; Kaushic *et al.*, 2000; Brabin, 2002). Studies indicate that uterine immune function is enhanced during the follicular phase and estrogen treatment enhanced uterine immune function in ovariectomized ewes, mares and cows (Washburn *et al*., 1982; Roth *et al*., 1983; Carson *et al*., 1988; Lander *et al*., 1990). Wulster *et al*. (2003) reported in gilts, enhanced susceptibility to uterine infections after intra-uterine challenge with *E. coli* and *Arcanobacterium pyogenes* during diestrus. Jason and Joseph (2008) reported that proestrous mice are protected from cardiovascular and immunological dysfunction following trauma-haemorrhage insult. Tibbetts *et al*. (1999) reported estrogen to have proinflammatory effects on neutrophil and macrophage infiltration in the mouse uterus during the estrous cycle. Several lines of investigation in humans suggest that disease expression is affected by the reproductive status and diseases like multiple sclerosis, asthma or systemic lupus erythematosis are exacerbated during specific periods of the menstrual cycle or pregnancy (Skobeloff *et al*., 1996; Case and Reid, 1998; Whitacre, 2002). Sartin *et al*. (2003) have shown that estrogen plus progesterone implants favourably alter the time course or disease severity of many of clinical manifestations associated with coccidiosis and endotoxemia in calves. Ansar *et al*. (1985) and Cutolo *et al*. (1995) maintained that androgens and progesterone exert suppressive effects on both humoral and cellular immune responses and seem to represent natural anti-inflammatory hormones whereas estrogens exert immune-enhancing activities.

Cytokine responses to provocative stress challenges modelled by endotoxin (LPS) administration, as well as active infection has received great attention as markers and mediators of both homeostatic and pathophysiological processes *in vivo*. Realising the probable influence of sex steroids in immune responses, the present study was planned to investigate variability in immune responses *in vivo* in cyclic buffaloes at estrus and diestrous stages of cycle in which distinctly different sex steroid milieu are maintained.

MATERIALS AND MERHODS

Ten apparently healthy female cyclic Murrah buffaloes of 1st parity were selected from the cattle research station of the institute and grouped into two E (estrous) and D (diestrous) with five buffaloes in each group. Group E had cyclic buffaloes at day 0 of estrous cycle whereas Group D at day 10 of estrous cycle. Stage of cycle in the buffaloes was confirmed through rectal examination by the concerned and well experienced veterinarian at the farm. All these Murrah buffaloes were maintained under routine management and nutritional practices as followed in the herd at the institute. Four animals from each group were given a single intravenous bolus of LPS (*E. coli* 055:B5 from Sigma Chemical Co., St. Louis, Missouri, USA.) 0.6 µg/kg body weight, in 10 ml sterile normal saline in the jugular vein. One animal serving as control from each group was administered 10 ml of sterile normal saline intravenously as placebo. The experiment was conducted in the morning in the month of September, with the average maximum temperature 30.5°C and the minimum 23.3°C. Before LPS challenge physiological parameters like rectal temperature, heart rate and respiration rate were recorded, and 6 ml of blood was drawn in heparinised vacutainers (Becton-Dickinson and Company, USA.) from the jugular vein in all

animals after taking all necessary aseptic measures. After LPS challenge, physiological parameters were recorded and 6 ml blood drawn in heparinised vacutainers, at 1, 2, 4, 8 and 24 h from all animals including control. During the whole experiment, the animals had free access to drinking water and fodder. The blood samples were transported to the laboratory on ice within 30 minutes. Total leukocyte count (TLC) and differential leukocyte count (DLC) were determined immediately after collection as described by Schalm *et al*. (1975), using Field stain for DLC. Plasma was separated by centrifugation and was stored at -20°C in aliquots of 0.5 ml in 1.5 ml micro-centrifuge tubes till analysis. The present experimentation was conducted in buffaloes after taking proper permission from the ethics committee of the Institute vide IAEC No. 23/09-21/11/2009.

Plasma nitric oxide was estimated as total nitrite (NOx) using a modified Griess reaction as described by Miranda *et al*. (2001). The test involved preparation of Griess-I (2% sulfanialamide w/v in 5% HCL), Griess-II (0.1% N-I-naphthyl ethylendiamine dihydrochloride w/v in Milli Q water) and vanadium chloride -III (VCl_3, 8 mg per ml of 1 M HCL). The chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Deproteinization of plasma was achieved by acetonitrile as described by Ghasemi *et al.* (2007). 100µl of each deproteinized sample and standard (sodium nitrite) was pipetted out in duplicates in a 96 well microtitre plate. 100 µl of VCl_3 reagent was added to each well, followed by 100µl of Griess reagent (Griess-I + Griess-II in 1:1 ratio) immediately. Incubation at 37°C for 30 minutes was carried out before absorbance was read at 540 nm wavelength in an ELISA plate reader (Microscan MS-5608A). The concentration was determined from the standard curve using a linear regression equation. Detection limit of NO was 1.56 µM. Inter assay and intra assay coefficients of variance were 3.4% and 4.62% respectively.

Plasma tumor necrosis factor α was evaluated by using a bovine tumor necrosis factor-α ELISA kit from Cusabio Biotech Co., Ltd. The sensitivity of assay was 0.05 ng/ml and inter assay and intra assay coefficient of variance was 6.25 and 5.4% respectively.

Plasma xanthine oxidase was estimated by using xanthine oxidase assay kit from Bio Vision Research Products, USA. The detection limit was 1 mU/100ml of reaction volume. Inter assay and intra assay coefficients of variance were 7.5 and 6.75% respectively.

Plasma cortisol was determined by using a cortisol EIA kit from Cayman. The sensitivity of the assay kit was 6.6 pg/ml. The inter assay and intra assay coefficients of variance were 6.7 and 6.25% respectively.

Sex steroids estrogen and progesterone were estimated by RIA using ^3H tracers. Antiserum for estradiol was procured from Sigma Chemical Co., St. Louis, Missouri, USA. whereas that for progesterone was a gift of Dr. B.S.Prakash. Progesterone in plasma was estimated by a direct RIA technique developed by Kamboj and Prakash (1993). Estrogen was extracted in benzene and counting of β-radiation was performed in a Beckman β counter, USA. Recoveries of estradiol and progesterone were 86% and 95%, respectively. Inter-assay and intra-assay coefficients of variance were 13.3% and 10.8% in estradiol and 9.4% and 10.25% in progesterone.

The data analysis was performed using a Systat 12 software package (Systat Software Inc 1735 Technology Dr., Ste.430, San Jose, CA 95110, USA). Analysis of variance of the data was performed using two way ANOVA with variables, group and time, included in the model as fixed

effects and Tukey's Honestly-significant difference test was employed. Values are presented as mean ± S.E. Graph and charts were prepared in Microsoft Excel 2007. Area under concentration x time curve (AUC) was calculated by commonly approached numerical approximation method called the trapezoidal rule.

RESULTS

In Group E buffaloes, the average estradiol levels were 10.41±0.61 pg/ml which differed significantly (P<0.01) from the 7.16±0.70 pg/ml registered in Group D buffaloes. The average plasma levels of progesterone in Group E and Group D buffaloes were 0.28±0.05 ng/ml and 2.21±0.13 ng/ml, respectively; the difference between the two groups was significant (P<0.01).

The important physiological parameter rectal temperature was recorded before LPS challenge (0 h) and at 1 h, 2 h, 4 h, 8 h and 24 h post challenge. Group E (estrous) buffaloes registered an average of 24 h rectal temperature significantly (P<0.01) higher (101.804±0.17°F) than Group D (diestrous) buffaloes (100.963±0.17°F) at LPS challenge. Temperature variation between the time intervals was statistically significant and peaked between 2 to 4 h after LPS challenge in both the groups. Subsequently rectal temperature declined to the normal 0 h levels beyond 8 h (Figure 1a). Integrated rectal temperature responses to LPS challenge calculated as AUC (area under curve) with baseline control subtracted for first 8 h was significantly (P<0.05) higher in Group E as compared to Group D Murrah buffaloes (Table 1.1).

Group E buffaloes registered 24 h mean heart rate 55.37±0.57 beats/min; this was not significantly different from the 54.41±0.51 beats/min in Group D at LPS challenge. However, the heart rate varied significantly (P<0.001) between the time intervals; it peaked at 1 h and started declining thereafter to baseline values (Figure 1b). Integrated heart rate responses to LPS challenge calculated as AUC with baseline control subtracted for the first 8 h was significantly (P<0.01) higher in Group D than Group E buffaloes (Table 1.1).

Group E buffaloes registered a significantly (P<0.001) higher 24 h mean respiration rate (26.45±0.34 breath/min) than Group D (24.41±0.34) at LPS challenge. Respiration rate varied significantly (P<0.001) between the time intervals; it started increasing soon after LPS challenge, reached its peak at 1 h (28.87±0.59), remained higher than baseline even at 8 h post challenge and reached normal baseline beyond 8 h (Figure 1c). Respiration rate responses to LPS challenge calculated as AUC with baseline control subtracted for first 8 h was not significantly different in Group E and Group D females (Table 1.1).

With respect to the immune mediators, Group E buffaloes registered a significantly (P<0.001) higher mean of 24 h plasma TNFα (2.079±0.07 ng/ml) than Group D (0.523±0.07 ng/ml) at LPS challenge. TNFα levels increased shortly after challenge, peaked at 2 h but then receded to 0 h level at 8 h post challenge in Group D but remained elevated beyond 8 h in Group E (Figure 2a). Integrated TNFα responses to LPS challenge determined as area under time x concentration curve (AUC) with baseline control subtracted for first 8 h was significantly (P<0.01) higher in Group E than in Group D buffaloes (Table 1.1).

Group E buffaloes registered significantly (P<0.001) higher 24 h mean plasma NO (39.20 ±0.61 μM) than Group D buffaloes (26.53±0.61 μM) at LPS challenge. Plasma NO levels increased

significantly (P<0.001) during the course of LPS challenge with levels reaching their peaks at 8 h post challenge (Figure 2b). Integrated NO response to LPS insult calculated as AUC with baseline control subtracted for 24 h was significantly (P<0.01) higher in Group E than Group D cyclic buffaloes (Table 1.1).

The mean plasma level of xanthine oxidase was not different between Groups E and D buffaloes amounting to 5.78±0.10 and 5.62± mU/ml, respectively, as an average of 24 h at LPS challenge. However, plasma XO levels varied significantly (P<0.001) between time intervals and registered peaks at 8 h after LPS challenge. Levels remained higher than 0 h even after 24 h of challenge (Figure 2c). Integrated XO responses to endotoxin challenge determined as AUC with baseline control subtracted for 24 h was significantly (P<0.001) higher in Group E than in Group D buffaloes (Table 1.1).

Group E buffaloes registered average 24 h plasma cortisol significantly (P<0.001) lower (1.30±0.02 ng/ml) than Group D (1.75±0.02 ng/ml) at LPS challenge. Plasma cortisol levels varied significantly (P<0.001) between the time intervals, registered peaks at 8 h, and then dropped to baselevel by 24 h post challenge. In Group D cortisol was significantly higher than in Group E from h 2 post challenge and remained high for 24 h (Figure 2d). Integrated cortisol responses to challenge calculated as AUC for first 8 h were significantly (P<0.001) higher in Group D than in Group E buffaloes (Table 1.1).

Buffaloes in Group E registered a 24 h mean plasma glucose level significantly (P<0.001) higher (58.80±0.80 mg/dl) than Group D females (53.33±0.80 mg/dl) at LPS challenge. Plasma glucose levels varied significantly (P<0.001) between the time intervals and registered hyperglycemia with peaks at 1 h after LPS challenge. Thereafter, the glucose levels receded below base level only at 2 h post challenge in Group D but not in Group E whereas buffaloes of both the groups registered hypoglycaemia at 4 h to 8 h post challenge; levels returned to normal baseline by 24 h (Figure 2e). Plasma glucose response to

Table 1. Integrated responses of various immune mediators calculated as area under time x concentration curve (AUC) after LPS challenge at different stages of reproductive cycle in Murrah buffaloes.

Parameters	Estrous (d 0)	Diestrous (d 10)
Rectal temperature 0-8 h (°F x h)	$16.43^{a} \pm 3.81$	$4.13^{b} \pm 2.01$
Heart rate 0-8 h (beats/min x h)	$20.625^{a} \pm 2.136$	12.875 ± 2.230^{b}
Respiration rate 0-8 h (breath/min x h)	20.500 ± 1.323^{a}	17.250 ± 2.016^{a}
TNFα for 0-8 h (ng/ml x h)	$11.297^{a} \pm 1.34$	$3.636^{b} \pm 0.28$
Nitric oxide for 0-24 h (µM/L x h)	$358.217^{a} \pm 25.07$	$224.962^{b} \pm 14.80$
XO for 0- 24 h (mU/ml x h)	$61.210^{a} \pm 4.17$	$32.360^{b} \pm 1.37$
Cortisol 0- 8 h (ng/ml x h)	$10.413^{b} \pm 0.32$	$14.862^{a} \pm 0.35$
Glucose 0-2 h (mg/dl x h) [Hyperglycaemia]	$22.091^{a} \pm 1.46$	$11.334^{b} \pm 0.90$
Glucose 2-8 h (mg/dl x h) [Hypoglycaemia]	$-25.707^{a} \pm 2.49$	$-43.345^{a} \pm 5.04$

Values in the same row with different superscripts differ significantly (P<0.05).

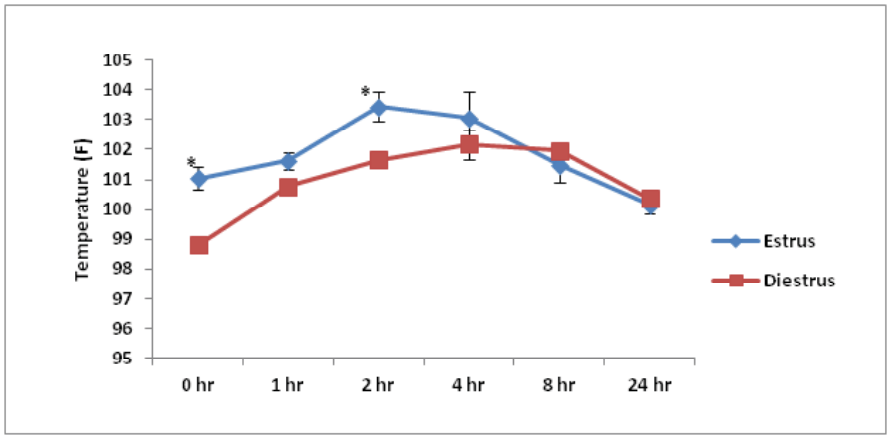


Figure 1a.

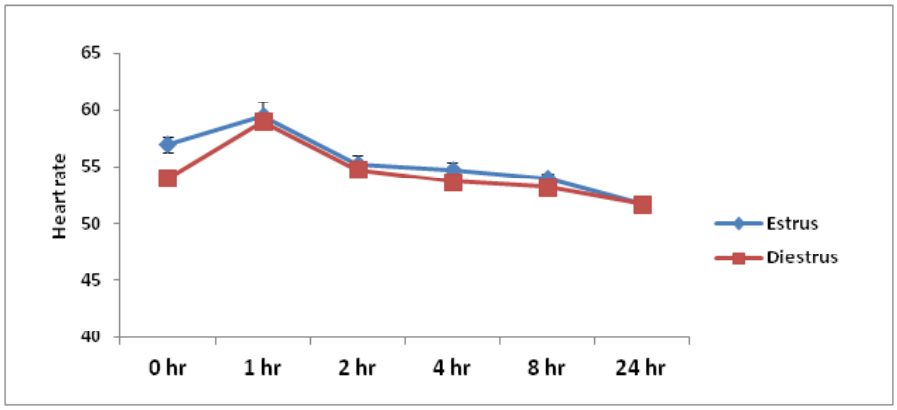


Figure 1b.

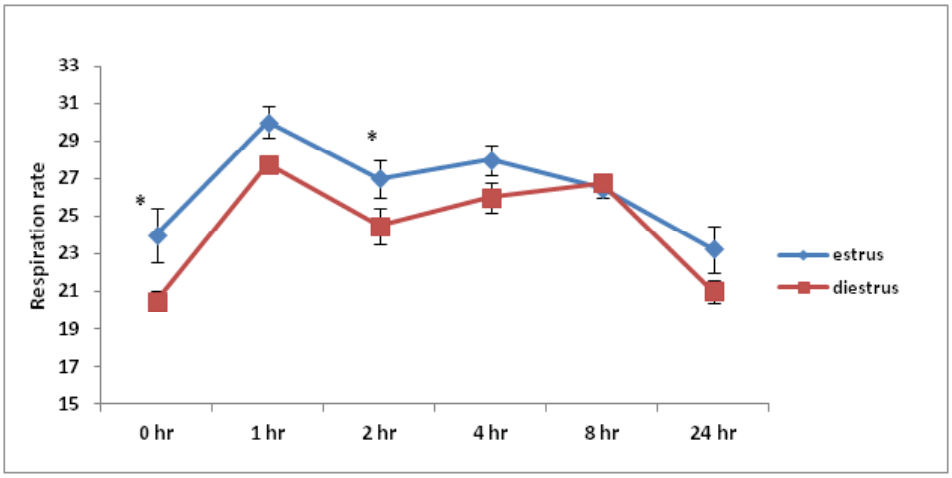


Figure 1c.

Figure 1a-c. Temporal changes in physiological parameters after LPS challenge in cyclic Murrah buffaloes. Data represents means ± SE (n= 10), *P< 0.05 at same point of time.

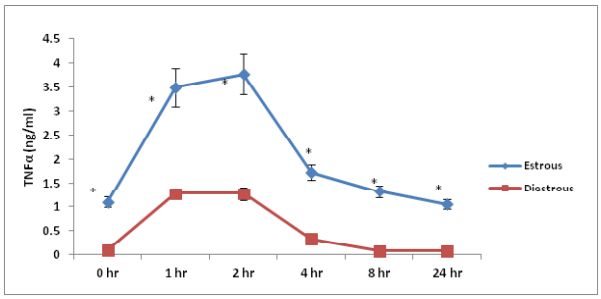
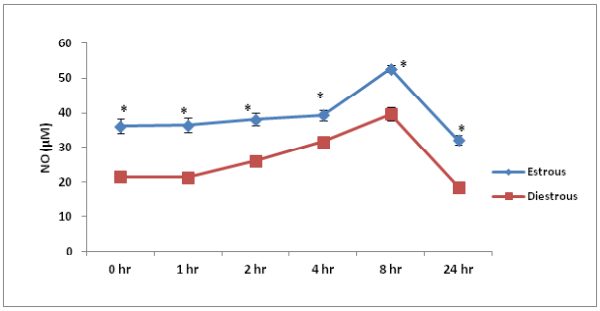


Figure 2a.



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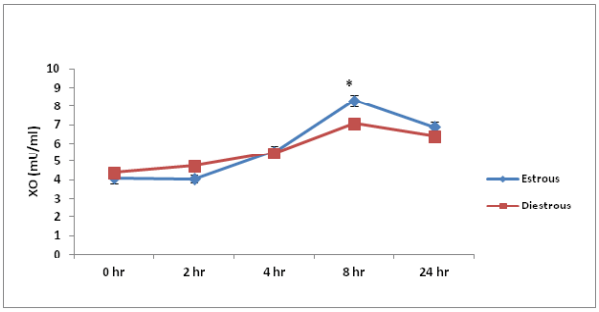

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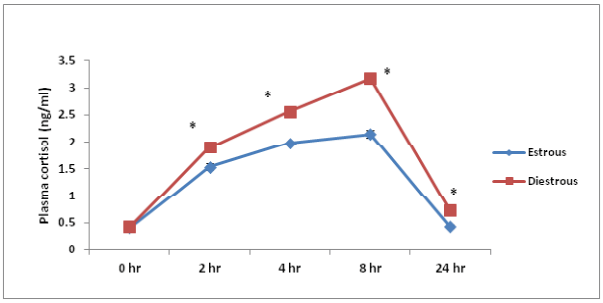

Figure 2d.

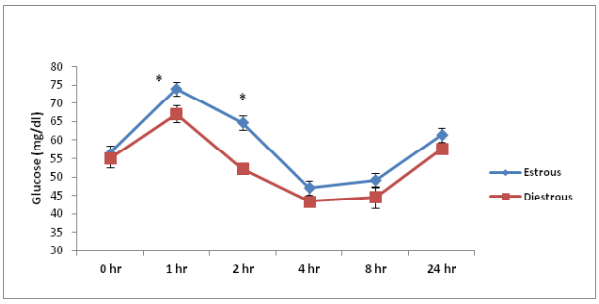

Figure 2e.

Figures 2a-e. Temporal changes in plasma TNFα (ng/ml), NOx (µM/L), XO (mU/ml), cortisol (ng/ml) and glucose (mg/dl) after LPS challenge in cyclic Murrah buffaloes. Data represent means ± SE (n= 10), *P< 0.05 at the same point of time.

LPS determined as AUC for 0 to 2 h and 2 -8 h of LPS challenge revealed hyperglycemia during the first 2 h with AUC significantly (P<0.001) higher in Group E buffaloes as compared to Group D buffaloes and hypoglycaemia from 2-8 h post challenge with AUC significantly (P<0.001) lower in Group D than Group E buffaloes (Table 1.1).

Buffaloes in Group E had a significantly (P<0.01) higher mean total leukocyte count ($12.25\pm0.31x10^3$) than Group D ($10.90\pm0.31x10^3$) at LPS challenge. However, there was no significant change in total leukocyte count between 0 h (11.30±0.31) and 24 h (11.85±0.31) after LPS challenge (Figure 3). Lymphocyte percentage did not significantly differ between Group E and D buffaloes. Group E and D buffaloes registered neutrophilia after LPS challenge with average neutrophil percentages of 22.250±0.80 and 25.00±0.08 at 0 h increasing significantly (P<0.01) to 29±0.80% and 28±0.80%, respectively, at 24 h after LPS challenge (Figure 3).

DISCUSSION

During the last two decades, sex hormones have become recognized as integral signalling modulators of the mammalian immune system. Endotoxin challenge in animal models to profess cytokine responses has received great attention as indicators for and mediators of both homeostatic and pathophysiological processes *in vivo*. Variability in the levels of immune mediators like TNFα, xanthine oxidase or nitric oxide between the stages of the reproductive cycle strongly suggest the role of sex hormones in immune-modulation, which is supported by Bouman *et al.* (2005). Immunological evidence suggests that female sex hormones play a role in the aetiology and course of chronic inflammatory diseases (Cutolo and Wilder, 2000).

The plasma estradiol levels were reasonably high in Group E and perceptible in Group D whereas plasma progesterone was far

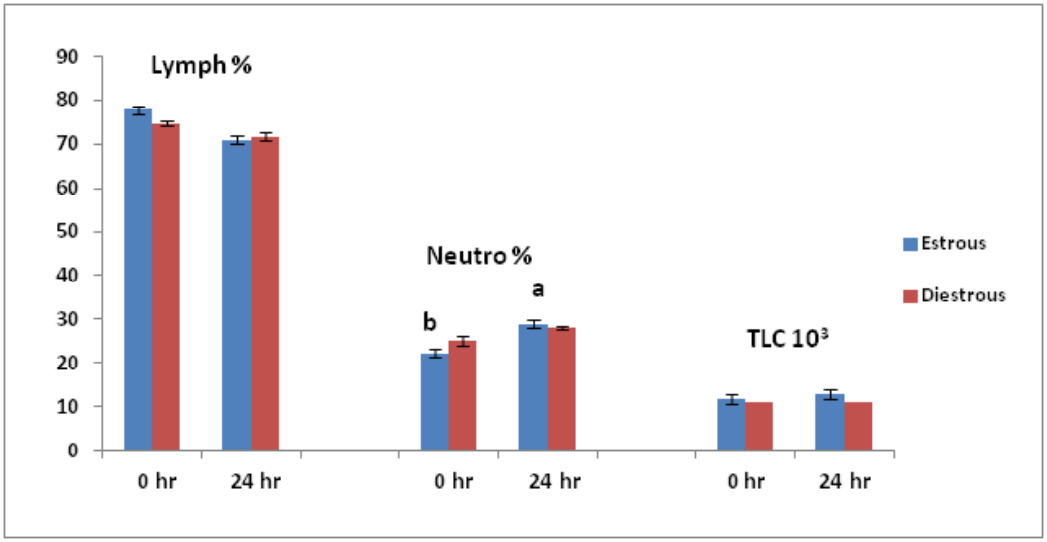

Figure 3. Changes in total leukocyte count (TLC) & differential leukocyte count (DLC) before and after 24 h of LPS challenge in cyclic Murrah buffaloes. Data represent means ± SE (n=10), superscript A & B or a & b differ significantly (P<0.05).

less in Group E than Group D buffaloes. Present findings were similar to the report of Shafie *et al*. (1982) who recorded peak levels of estradiol (20 pg/ml) during follicular phase and 16.7 pg/ml on day 11of estrous cycle. Since blood sampling was done after the onset of estrus , the levels of estrogen observed in present investigation could not have reflected peaks. However, the sex steroid levels observed in present study were comparable to the levels reported by Sartori *et al*. (2004) in Holstein Frisian heifers, Dhali *et al*. (2006) in mithun and Kanai and Shimizu (1984) in swamp buffaloes. The progesterone levels in Group D and Group E buffaloes were lower than the levels reported by Shafie *et al*. (1982) in buffaloes but closely approximated the levels reported by Rao and Pandey (1982) in buffaloes, Dhali *et al*. (2006) in mithun and Mondal and Prakash (2003) in Sahiwal cows. The present study demonstrated that Group E and Group D buffaloes had distinctively different sex steroid hormone backgrounds.

Group E buffaloes reported higher values for physiological parameters at endotoxin challenge as compared to Group D indicating dimorphism in responses to endotoxin in cyclic females during estrus and diestrous characterised by different sex steroid milieu. The Group E buffaloes were, therefore, moderate responders and Group D low responders. Since the estrogen is believed to enhance the immune reactions (Jason and Joseph, 2008; Tibbetts *et al*., 1999), Group E animals had estrogen as a predominant sex steroid present in circulation, and this could probably have been responsible for heightened physiological responses to endotoxin challenge as compared to Group D animals with progesterone as a major circulatory sex steroid. In the present study, Group E females registered higher levels of immune response mediators than Group D females,which is in agreement with the findings of Kahl *et al*. (2009) in cattle, Horadagoda *et al*. (2002) in buffaloes, Michie *et al*. (1988) in humans, and Schlafer *et al*. (1994) in sheep. Due to variations in sex steroid levels with the stage of estrous cycle, estrogen and progesterone might be candidates for hormones regulating immune responses to LPS challenge. Plasma TNFα levels were perceptibly low at all time points in Group D buffaloes as compared to Group E; however, the TNFα levels elevated immediately after LPS challenge and were at peak after only 1 h in both the groups of animals, but TNFα levels remained significantly higher than 0 h level for a longer duration in Group E buffaloes as compared to Group D. TNFα, a primary inflammatory cytokine produced by immune cells, governs the secretion and cascade of other cytokines involved in immune reactions to antigenic challenge, and the outcome of disease is, therefore, largely monitored by this cytokine. Buffaloes at estrus with high TNFα may counter the challenge effectively by involving other immune mediators or cytokines but at the same time animals might get exposed to pathophysiological risks also. Similarly, NO was higher in Group E buffaloes at every point of time than Group D and peaked at 8 h post challenge in both the groups. Whereas XO peaked at 8 h post challenge and was significantly different in Group E than in Group D buffaloes. LPS challenge resulted in increased circulatory levels of XO as well as NO, which both play an important role in immune defence and homeostasis. XO participates in the activation of systemic inflammatory cells such as increased adherence and/or rolling of neutrophils to the endothelium, which, if not balanced, leads to lung injury with poor prognosis. However, at the same time, elevated nitric oxide counteracts XO functions by reducing P-selectins, and thus helps in the maintenance of homeostasis (Lance *et al*.,

1997).

A good quantity of investigation supports the view that progesterone suppresses immunity and increases susceptibility to infections as reported by Ramadan i. (1997) in sheep, White *et al*. (1997) in humans , Kaushic *et al*. (2000) in mice, Wulster-Radcliffe *et al*. (2003) in gilts and Kahl *et al*. (2009) in cattle. Progesterone, referred to commonly as immunosuppressive, was supported by the present study and probably may be a requirement for reception and attachment of an embryo in the uterus and or a protection from its rejection. The exact mechanism by which the progesterone suppresses immunity is not well understood; however, recently Li *et al*. (2009) reported that progesterone inhibited immune response to lipopolysaccharide by modulating Toll-like receptor (TLR) signalling and inhibited TLR4 and TLR9-triggered IL-6 and nitric oxide production in macrophages, significantly inhibited LPS-induced nitric oxide synthase (iNOS), and up-regulated expression of suppressor of cytokine signalling (SOCS1) protein. Reports suggest that anti-inflammatory properties of progesterone in rodents and humans are mainly mediated through inhibition of production and release of a number of proinflammatory cytokines and inhibition of NO production (Miller and Hunt, 1996). The anti-inflammatory steroid cortisol was, however, reported high and sustained in Group D buffaloes as compared to Group E and these findings are comparable to the findings of Schlafer *et al*. (1994), Kahl *et al*. (2009). Probably estrogen, a predominant sex steroid in Group E buffaloes favoured production of proinflammatory cytokines whereas progesterone, predominant in Group D buffaloes, disapproved it but favoured production of the anti-inflammatory hormone cortisol. This anti-inflammatory property of progesterone is of special importance during pregnancy (Druckman and Druckman, 2005). The overall frail responses of immune mediators to endotoxin challenge in Group D buffaloes could probably be the handiwork of progesterone, which predominates during diestrous.

White blood cells are indispensible cellular components of two important arms of the immune system, the cell mediated and humoral immunity. In the present study, the shifts in the counts of two major cells with neutrophilia was evident after LPS challenge in both the groups of buffaloes. Any type of stress is recognised by neutrophilia (Pampori *et al*., 2010), and in the present study, neutrophilia suggested that the buffaloes were under stress due to LPS challenge.

The present study revealed that the variability in immune response mediators were present as a function of endocrinological character of the two stages (estrous and diestrous) of the estrous cycle. Therefore, sex hormone balance remains a crucial factor in the regulation of immune and inflammatory responses and the therapeutical modulation of this balance should represent a part of advanced biological treatments for many diseases. Further, the variability in immune responses inherent in female subjects at different stages of estrous cycle could affect the outcome of interventions.

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