

Alterations in polymorphonuclear leukocyte functions during periparturient period in buffaloes

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ABSTRACT

The study was planned to evaluate polymorphonuclear leukocyte (PMNL) functions in normally calved and dystocia affected buffaloes. Normally calved buffaloes (6) were blood sampled for 7 days around calving and 14 dystocia affected buffaloes were sampled from the day of delivery by obstetrical means to day 3 post delivery. Plasma cortisol concentration, total and differential leukocyte counts and PMNL phagocytosis and killing abilities were evaluated. In dystocia affected buffaloes, plasma cortisol was significantly higher than of normally calved buffaloes on all the days of observation and the neutrophil counts were significantly lower during the immediate postpartum period. In normally calved buffaloes, the changes in PMNL phagocytosis around calving were not significant. The percentage extra cellular killing activity decreased from prepartum to postpartum period. In dystocia affected buffaloes, the phagocytic activity was nonsignificantly higher whereas killing capacity was significantly lower as compared to normally calved buffaloes on the day of calving. In conclusion, this study revealed derangement in PMNL functions around calving that predispose the buffaloes to postpartum infections.

Key words: Buffalo, Dystocia, Leucocytes, Parturition

The changes in glucocorticoid (GC) levels around parturition alter the leukocyte counts (Tizard 1998, Ferguson and Hoenig 2001). The polymorphonuclear leukocytes (PMNL) are the major constituents of the granulocytic leukocyte population which play a central role in early defense against most bacterial infections (Burvenich *et al.* 2003). During the periparturient period, animals undergo marked physiologic changes that cause suppression of host defence mechanism and an increase in susceptibility to uterine and mammary gland infection (Hussain 1989). The stress hormones at parturition and during the first weeks of lactation have been proposed to partly contribute to the impaired function of PMNL (Diez-Fraile *et al.* 2003). The reduction in the plasma concentration of anti-oxidants (Michal *et al.* 1994), negative energy and protein balance and major changes observed around periparturient period in sex steroid hormone (Lamote *et al.* 2004) and eicosanoid levels (Kehrli *et al.* 1989, Hoedemaker *et al.* 1992a) may also contribute for reduced neutrophil activity.

Kehrli *et al.* (1989) found high ingestion activity by the PMNL during the periparturient period with slight decrease after parturition but Hoedemaker *et al.* (1992b) and Cai *et al.* (1994) observed high ingestion activity around parturition with further increase after parturition. Due to paucity of such studies on PMNL functions around parturition in buffaloes, the present study was planned to evaluate the ingestion and killing capacity of peripheral PMNL in buffaloes during the periparturient period involving normal and obstructive parturitions.

MATERIALS AND METHODS

Animals and treatments: Buffaloes (20) were divided into group 1 (6 Murrah buffaloes of the University dairy farm and calved normally) and group 2 included 14 Murrah buffaloes presented at Veterinary Clinics of the University for the treatment of dystocia. These animals were given the necessitated obstetrical [either mutations, fetotomy, detorsion by Sharma's modified Schaffer's method (Singh and Nanda 1996) or caesarean section] and supportive (antibiotics, saline and ecobolics) treatment. The normally calved buffaloes had a normal puerperium while the dystocia affected buffaloes suffered from retention of fetal membranes and/or postpartum metritis and were treated appropriately.

Sampling schedule: Blood samples were collected by

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jugular venipuncture from normally calved buffaloes daily from day 3 prepartum to day 3 postpartum and from dystocia affected buffaloes daily from the day of delivery by obstetrical means to day 3 post-partum using heparin as anticoagulant. The samples were transported to the laboratory on ice. An aliquot of blood was centrifuged immediately at 500 g for 15 min and the plasma was frozen at -20°C until analyzed for cortisol. Another aliquot was used for leukocyte counts within 2 h and the rest for PMNL isolation.

Radio immunoassay of cortisol: Plasma cortisol was estimated by radio immuno assay (Prakash and Madan 1984). The assay sensitivity was 0.2 ng/ml. The inter- and intra-assay coefficients of variation were 10.7 and 9.2%, respectively.

Leukocyte counts: Total leukocyte counts (TLC; using haemocytometer) and differential (DLC: Leishman's staining; battlement method of counting) were done using standard procedure (Jain 1986). Stained smears were examined for evaluating the maturity of neutrophils on different days postpartum.

Evaluation of phagocytic and killing activity of blood PMNL

Isolation of blood PMNL: The blood PMNL were isolated as per Zigmond and Hirsch (1972) modified by Strzemiński *et al.* (1984). Heparinized blood was placed in upright capped test-tubes and the red blood cells (RBCs) were allowed to sediment for 50 min at room temperature. 8 ml upper leukocyte rich fraction of plasma was layered over 3 ml histopaque and centrifuged at room temperature for 30 min at 200 g. The pellet was suspended in 2 ml of normal saline. RBCs were lysed by the addition of 6 ml distilled water for 20 sec. Isotonicity was restored by adding 2 ml of 3.6% saline. The suspension was centrifuged for 7 min at 100 g (room temperature). The pellet was resuspended in 6 ml of saline and allowed to stand for 15 min at room temperature. Centrifugation was repeated for 7 min at 100 g and the pellet resuspended in Hank's balance salt solution (HBSS) for counting with haemocytometer.

Viability testing and counting of isolated PMNL: The viability of the isolated PMNL was assessed using the Trypan Blue dye exclusion procedure described by Gupta (1992) which was around 80%. The PMNL were adjusted to a concentration of 1×10^7 viable PMNL/ml HBSS for the phagocytic assay.

Blood PMNL phagocytosis and killing: Phagocytosis and the killing ability of blood PMNL were estimated according to Metcalf *et al.* (1985) with slight modifications. Briefly, 0.5 ml of a solution containing 10^7 PMNL/ml, 0.3 ml fetal calf serum and 0.3 ml HBSS containing 4×10^8 *C. albicans*/ml were mixed in a sterile test tube. A second sterile test tube, which contained the same concentration of *C. albicans* and serum, was taken and used as control using HBSS instead of PMNL. Both tubes were incubated at 37°C . After 30 min, 0.1 ml mixture from the first test tube was transferred to

another sterile test tube containing 1 ml of 0.4% Trypan Blue in PBS at 4°C . A wet mount was made and 100 PMNL were counted to determine the percentage phagocytosis.

The first 2 test-tubes were incubated for another 45 min (total 60 min). To stop the reaction, 0.25 ml of 2.5% sodium deoxycholate was added. After the tubes were vortexed, 4 ml of 0.01% methylene blue in PBS was added, vortexed again and centrifuged at 2500 rpm for 15 min; 4 ml of the supernatant was discarded. To determine the percentage of extra cellular viable (unstained) and dead (stained) *C. albicans*, a total of 300 *C. albicans* were counted in haemocytometer and the percentage of organisms killed by blood PMNL was estimated using the following equation:

$\% \text{ C. albicans killed by blood PMNL} = \% \text{ killed (experimental)} - \% \text{ killed (control)}$.

Statistical analysis: Data are presented as mean $\pm$ SE. They were analyzed by Statistical Package for social sciences software programme. Significant differences between mean values within and between groups were identified using the Duncan multiple range test and independent samples t-test, respectively.

RESULTS AND DISCUSSION

Plasma cortisol: In group 1, the cortisol concentration on day 0 was significantly higher ($P < 0.01$) than all prepartum and postpartum concentration (Table 1). The initial cortisol concentration on day '0' in group 2 was significantly higher ($P < 0.01$) than in group 1. Within the group, the concentration on day 0 in group 2 was significantly higher ($P < 0.001$) than those on other days postpartum.

Leukocyte count: In group 1, the TLC on day 3 prepartum gradually increased to reach a peak on day '0' (Table 1). The counts declined nonsignificantly from day 0 to day 3 in both the groups. The TLC in group 2 was nonsignificantly lower than in group 1 on all the days of observation.

In this study, a prepartum rise, followed by a peak on the day of calving and then a postpartum fall in TLC followed the trend of plasma cortisol. Since the expression of adhesion molecule on neutrophils is diminished by glucocorticoids (Tizard 1998), increased demargination of neutrophils resulting in neutrophilia, occurs at the time of parturition due to higher cortisol level and this accounts for higher TLC at parturition (Ferguson and Hoenig 2001). The decrease in TLC during the immediate postpartum period could be associated with migration of neutrophils towards uterine lumen and the mammary gland (Guidry *et al.* 1976).

Higher blood histamine concentration (Matharu and Prabhakar 1999) and uterine bacterial contamination leading to endotoxaemia (Singh 2002) might have contributed to the reduced TLC in group 2 as compared to group 1, though cortisol concentration was significantly higher. Moreover, as the duration of dystocia increased, the discharge from the uterus became purulent on the day of delivery due to dead and emphysematous foetus with a very high count of

Table 1. Plasma cortisol concentration and leukocyte counts (mean±SE) in normally calved (group 1) and dystocia affected (group 2) buffaloes

Parameters	Groups	SL	Days around parturition						
			-3	-2	-1	0	1	2	3
Plasma cortisol (ng/ml)	1	0.01	2.49±0.35 ^a	4.50±0.56 ^{ab}	6.52±1.41 ^b	11.08±1.78 ^{c**}	5.33±0.94 ^{ab**}	3.88±0.75 ^{ab*}	2.71±0.68 ^{ab*}
	2(10)	0.001	-	-	-	41.50±7.73 ^{a**}	28.70±6.05 ^{b**}	21.02±6.51 ^{b*}	19.62±6.19 ^{b*}
TLC (× 1,000/cumm)	1(6)	NS	8.15±1.44	8.10±1.74	8.62±1.55	10.64±1.26	8.37±1.49	8.45±1.34	7.39±1.38
	2(14)	NS	-	-	-	6.18±1.47	3.65±0.75	3.66±0.86	4.15±0.84
Meta-myelocytes (× 1,000/cumm)	2(8)	NS	-	-	-	1.04±0.16	0.83±0.15	0.53±0.12	0.47±0.09
Band cells (× 1,000/cumm)	2(8)	NS	-	-	-	3.52±1.24	1.54±0.67	1.02±0.23	0.88±0.22
Mature neutrophils (× 1,000/cumm)	2(8)	NS	-	-	-	0.38±0.34	0.00±0.00	0.01±0.01	0.01±0.01

SL, Significance level within a group, NS, not significant; ^a, ^b and ^c differ significantly within a group; * and ** denote P<0.05 and P<0.01, respectively, between groups. Figures in parentheses indicate number of animals.

neutrophils per unit volume of the discharge (results not shown), indicating the drainage of neutrophils into the uterus. The common consequences of dystocia, viz. retention of fetal membranes (RFM) and/or metritis invariably occurred in group 2. These conditions result in shift of cells from the circulatory pool to the marginating pool and movement into the uterus (Cai *et al.* 1994), which might cause further reduction in TLC on subsequent days postpartum.

In group 1, the leukogram on the prepartum days of observation constituted only mature neutrophils. On the day 0 and 1 mature neutrophils with negligible number of band cells (results not shown) were observed. Subsequently, the leukogram reverted back to that of prepartum period. Leukograms of 8 buffaloes of group 2 were evaluated. Band cells dominated the leukogram, followed by metamyelocytes and very few mature cells on day 0. Immature neutrophils declined nonsignificantly during the postpartum period of observation.

The observation of mature neutrophils dominating the leukogram with negligible number of immature forms in group 1 was in line with that of Mateus *et al.* (2002) in normally calved cows. The left-shift to the myelocyte stage

observed in group 2 depicted endotoxin-induced changes (Taylor 2000). The degenerative left-shift with myelocytes and band cells was due to intense mobilization of cells from the bone marrow reserve pool (Taylor 2000). In the present study, RFM, metritis and the dystocia associated inflammatory changes probably resulted in the drainage of neutrophils into uterus with subsequent mobilization of immature cells from reserve pools resulting in the left-shift of neutrophils. Hence, it may be inferred that the inflammation and infection accompanied with dystocia could overcome the effects of even the highly elevated endogenous cortisol.

PMNL functions

The neutrophils were significantly lower (P<0.01) in group 2 as compared to group 1 on all the days postpartum. This limited the valuation of PMNL functions only on day 0 only in 13 buffaloes. In group 1, though changes in phagocytizing ability of blood PMNL were not significant around parturition, percentage phagocytosis increased from 2–3 days prepartum to reach higher levels on day 0 followed by a decline to prepartum values by day 2 to 3 postpartum. In group 2, the phagocytic activity on day 0 was

Table 2. PMNL functions (mean±SE) in normally calved and dystocia affected buffaloes

Parameters	Groups	Days around parturition						
		-3	-2	-1	0	1	2	3
Phagocytosis (%)	1(6)	13.13±0.13 ^a	12.1±0.11 ^a	18.88±5.62 ^a	23.32±4.74 ^{a1}	17.99±5.06 ^{a/1}	12.57±2.78 ^a	12.21±0.21 ^a
	2(13)	-	-	-	30.82±2.69 ¹	-	-	-
Extracellular killing (%)	1(6)	16.98±0.23 ^a	12.40±0.83 ^{ab}	11.17±0.31 ^{ab}	10.63±1.10 ^{ab/1}	8.74±0.79 ^{ab}	9.18±4.42 ^{ab}	5.24±1.60 ^b
	2(13)	-	-	-	4.09±1.32 ²	-	-	-

^a and ^b significantly different between rows (P<0.01); ¹ and ² significantly different between the columns (P<0.05). Figures in parentheses indicate number of animals.

nonsignificantly higher than that in group 1. In group 1, the killing percentage gradually declined from day 3 prepartum to reach a significantly lower ($P < 0.01$) percentage on day 3 postpartum. In group 2, the killing percentage on day 0 was significantly lower ($P < 0.05$) than that of group 1.

Increase in the ability of blood PMNL to ingest bacterial or yeast cells around parturition observed in the present study was in agreement with earlier reports (Kehrli *et al.* 1989, Hoedemaker *et al.* 1992b, Cai *et al.* 1994). Increased ingestion capacity might be necessary mechanism to prevent the infections in the periparturient period (Hoedemaker *et al.* 1992b). Increased cytokines (CK), prostaglandin (PG), and acute phase proteins (APP) at the time of parturition (Lewis *et al.* 1984, DelVecchio *et al.* 1994, and Sheldon *et al.* 2001) are capable of augmenting PMNL functions (Kelley *et al.* 2001). Though the increased blood cortisol concentration is immunosuppressive (Andreasen and Roth 2000) the combined effects of PGs, CK and APP around parturition might result in increased ingestion capacity of blood PMNL. Markedly higher plasma PGFM concentrations and APP response (Scott *et al.* 1992) in dystocia affected animals could be responsible for the increase in phagocytosis in them. The high phagocytic activity despite the presence of more immature neutrophils suggested no significant difference between the functions of mature and immature neutrophils (Andreasen and Roth 2000).

The extra cellular killing by PMNL is an antibody dependent (ADCC) or independent cell mediated cytotoxicity, which is referred to as a form of neutrophil degranulation with extracellular release of cytotoxic substances (Hoedemaker *et al.* 1992a). The neutrophil is the most efficient bovine cell capable of mediating ADCC. The decline in killing activity of PMNL observed during the periparturient period in the present study was in line with the earlier observations in cows by Guidry *et al.* (1976), Cai *et al.* (1994) and Hussain and Daniel (1992). This decrease could be due to impairment of PMNL functions at this time (Hoedemaker *et al.* 1992b).

Onset of hormonal response to stress is rapid and thus stress induced alterations in immune functions are observed within a short time (Magnusson *et al.* 1998). The ability to mediate ADCC was depressed in neutrophils taken from GC or adrenocorticotrophic hormone treated cattle (Andreasen and Roth 2000). Yet, the mechanism by which steroids change the immune response is poorly understood (Lamote *et al.* 2004). However, it has been partially explained by interference of GCs with the transcription of genes for cytokines. GCs have been reported to decrease the ascorbic acid content of neutrophils and thus inhibit the neutrophil functions (Roth *et al.* 1982). Suppression of neutrophil ADCC activity observed after treatment with GCs in cattle was apparently mediated through their effects on monocytes which subsequently released factors altering neutrophil functions (Andreasen and Roth 2000). Hence, higher levels

of cortisol around parturition could be attributed for suppressed killing activity of PMNL.

It is paradoxical that ingestion capacity increased but the more important killing ability of PMNL decreased simultaneously around parturition when defensive mechanism of PMNL were strongly required to combat infections. Although ingestion capacity of PMNL is increased at parturition, the bacterial mechanism were found to be impaired thereby rendering the animal more susceptible to bacterial infections (Hoedemaker *et al.* 1992b, Mateus *et al.* 2002).

In conclusion, the study suggested that significant changes occur in leukocytic functions around parturition and dystocia suppressed killing ability of the leucocytes, thus predisposing the animals to postpartum infection.

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