The Indian Journal of Animal Reproduction, 24(1): 9-15, June 2003

RESEARCH ARTICLE

T-lymphocyte profile as a predictive marker for placental retention in buffalo (*Bubalus bubalis*)

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Received: October 6, 2001
Accepted: May 23, 2002

ABSTRACT

Peripheral blood lymphocyte profile was assessed in buffaloes with normal placental separation (n=5) and those with retention of placenta (n=5). Jugular venous blood samples (30 ml) were collected from every buffalo on 1 to 10 days pre partum, day of calving and on 1 to 10, 15, 20, 25 and 30 day post partum. T-lymphocyte profile was evaluated, with respect to, per cent peripheral blood lymphocyte sheep red blood cell-rosettes (SRBC-R). Plasma vitamin-A, b-carotene, vitamin - E and zinc were estimated for every subject on the sampling days, by standard methods. Significant ($P<0.01$) differences in the mean values of SRBC-R per cent were observed between subjects with normal placental separation and those with retention across the respective days. SRBC-R per cent and plasma parameters indicated a steady decline from 10 to 1 day pre partum, the declination being significant ($P<0.01$) around 6 to 4 day pre partum till day of calving in both the groups, whereas, the values were significantly lower ($P < 0.01$) in affected group. Lower values persisted for 5 to 10 day post-partum in control group and for 20 to 25 day post partum in affected group. Significant ($P < 0.01$) relationships between SRBC-R profile and plasma biochemical variables were seen. The overall effect of vitamin A, b-carotene, vitamin E and zinc was statistically significant ($P < 0.01$) in both the groups. The results suggest that better the initial state of health of the buffaloes, in terms of their SRBC-R status, better the pregnancy outcome. Thus concluding that retention of placenta is associated with abnormally low levels of peripheral blood T-lymphocyte levels at calving. Also that the pre partum lower ($P<0.01$) T-lymphocyte profile in retention cases may serve as an index of retention of fetal membranes in buffaloes.

Key Words: Retained Placenta, T-lymphocytes, vitamin A, β-carotene, vitamin E, zinc, buffalo.

Retention of the placenta, an undesirable sequel of parturition, is common in buffaloes (Pattabiraman and Baura, 1977, Khar, 1980) with subsequent ill effects on the fertility and milk production. Low serum β-carotene (Gul and Timurkan, 1989), vitamin E (Julien *et al.*, 1976 a & b) and zinc (Zhang *et al.*, 1992) have been suggested as predisposing factors for retained placenta in dairy cows and buffaloes (Singh *et al.*, 1997). The etiology of retained placenta has been reviewed in bovidae (Wetherhill, 1965, Roberts, 1971 and Mueller *et al.*, 1989) but the specific cause is still unclear, there may be an immunological determinant (Heuwieser and Gruner[4], 1987, Joosten, 1990).

Studies on the peripheral blood thymus-dependent lymphocytes (PBL-T), primarily concerned with the transplant rejection are of interest at the time of parturition and placental shedding. Whereas, although the PBL-T profile during prgenancy in human and other animal species has been studied, the results have been contradictory (Bulmer and Hancock, 1977, Bolis *et al.*, 1979, Cornfield *et al.*, 1979), no such studies have been reported around parturition and in puerperal disorders in buffaloes. The relationship of plasma vitamin A, β-carotene, vitamin E and zinc with PBL-T during the peripartum period is unknown in buffaloes calving normally or those retaining their after births. We hypothesized that these biochemical variables would show a positive relationship with PBL-T and thus might explain protective effect against puerperal affects leading to placental retention. Therefore, using a

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prospective comparative design, buffaloes with, and without, placental retention were assessed peripartum for PBL-T profile by erythrocyte rosetting technique.

MATERIALS AND METHODS

Test buffaloes and collection of blood samples

Ten pluriparous (≥3 calvings) pregnant Murrah buffaloes, at the Dairy Farm of the Punjab Agricultural University (PAU), Ludhiana, India, were used in this study. Five normally calving buffaloes, which shed their after birth within 8 hours of calving, comprised the normal control group, whereas, five buffaloes retaining their placentae for more than 12 hours post partum comprised the affected group. The buffaloes, raised under loose house system in the present study, were maintained under standard managemental conditions. Placenta in the affected group was removed manually after 12 hours post partum and antimicrobials based on culture sensitivity of uterine microflora were infused intrauterine for 5 days post partum.

Jugular venous blood samples (30 ml) were collected on 10 to 1 day pre partum, day of calving and 1 to 10, 15, 20, 25 and 30 day post partum in all the subjects. Plasma was separated by centrifugation (2000 rpm for 5 min) and stored at -20°C.

Peripheral blod lymphocyte sheep RBC rosetting

Sheep RBC (SRBC) were treated with 2-amino ethyl isothiouronium bromide hydrobromide (AET, 0.14 M, pH 9.0, Sigma Chemicals co, USA) solution in normal saline (Madsen and Johnsen, 1979). One per cent suspension of SRBC-AET was prepared in RPMI-1640 tissue culture medium (without fetal calf serum), stored at 4°C and used within 6 days for rosette assay. PBL-were isolated from heparinized venous blood, by Ficol Hypaque (FH) gradient (Sigma Chemicals Co., USA) (Boyum, 1968). A final concentration of 5 x 10^6 cells per ml in RPMI-1640 (without fetal calf serum) was made with 97-98 per cent viable PBL. SRBC-R were prepared (Bentwich et al., 1973), with slight modification, 0.2 ml each of 1 per cent SRBC-AET and PBL (5 x 10^6 cells/ml) suspension, were mixed thoroughly and incubated at 4°C for six hours. PBL binding with 3 or more SRBC-AET were considered as SRBC-R.

Biochemical analysis

Principle of Carr - price reaction was used for the detection of plasma vitamin A and b-carotene levels (Kimble, 1939). Colorimetric method by Bolliger *et al.* (1956) was applied for vitamin E estimation in plasma. Plasma zinc concentrations were estimated by the Atomic Absorption Spectrophotometry (Ludmilla, 1976).

Statistical analysis

The analysis of data were done by applying ANOVA taking complete randomized block design (CRD) with unequal number of replications (Snedecor and Cochran, 1980). Regression analysis was done by fitting the multiple linear regression equation.

RESULTS AND DISCUSSION

Peripheral blood lymphocyte sheep RBC rosetting (SRBC-R)

Significantly ($P < 0.01$) lower percentages of T-lymphocytes from buffaloes with retained fetal membranes formed SRBC-R, with sheep erythrocytes, compared with those which shed the placenta normally (Table 1). The mean ± SEM per cent SRBC-R, starting day 10 pre partum (33.50±0.39 in control and 32.00±0.46 in affected) till day of calving (6.70±1.38 in control and 2.62±0.1 in affected), declined in both control and affected groups. No marked differences were observed between the two groups till day 6 pre partum (23.60±0.29 in control v/s 23.13±0.40 in affected, P > 0.10). From day 5 before calving the per cent SRBC-R (21.20±1.08 in control v/s 11.88±0.13 in affected, $P < 0.01$), in affected group differed markedly ($P < 0.01$) from those in the control group.

In the post partum period (Table 2), the per cent SRBC-R differences in the control and affected groups were significant ($P < 0.01$). In buffaloes with placental retention the count rose from 4.15±1.0 per cent to 6.30±0.34 per cent and finally to 32.50±0.50 per cent from day 1 to day 5, and to day 15 post partum, correspondingly. In the control group animals low counts were maintained till day 5 post partum (16.95±0.43 per cent) showing marked increase by day 10 post partum (44.40±0.1 per cent). By day 15 post partum the values were almost normal (52.40 ±0.18 per cent) in control group, whereas, in the affected animals similar levels were attained by day 30 post partum (52.00±0.25 per cent).

Plasma biochemical variables: vitamin A, carotene, vitamin E and zinc measured in µg per decilitre for pre partum and day of calving and post partum period are given in Table 3 and respectively. The levels of these variables in affected group were lower as compared to cont

Table 1: Pre partum and calving per cent PBL - SRBC-R (Mean±SEM) for normal and affected buffaloes

Period	Day	Groups	
		Normal (n=5)	Affected (n=5)
Pre partum	10	33.50±0.39	32.00±0.46
	9	29.75±0.19	28.75±0.20
	8	29.55±0.43	28.50±0.40
	7	27.60±0.48	27.38±0.20
	6	23.60±0.29	23.13±0.40
	5	21.20±1.08	11.88±0.13
	4	21.10±0.98	9.87±0.13
	3	12.15±0.37	6.63±0.19
	2	11.80±0.08	5.13±0.05
	1	10.30±0.30	4.88±0.06
Calving	0	6.70±1.38	2.62±0.11
CD between days (P<0.05)		2.14	0.80
CD between group (P<0.01)		1.59	

group across the respective days. The values indicated a decline starting day 10 pre partum to day of calving in both the groups. In the post partum period the levels in the buffaloes suffering from placental retention were restored at later stage than in control group.

Relationship of plasma biochemical variables with PBL-SRBC-R

a) Pre partum and at the time of delivery

Significant positive correlation was present between per cent SRBC-R and biochemicl variables: vitamin A (n = 55, r = 0.429, P < 0.05), β-carotene (n=55, r =0.881, P<0.01), vitamin E (n = 55, r = 0.962, P<0.01) and zinc (n=55, r=0.732, P< 0.01). Similarly, in control group significant positive correlation (n=55, P<0.01) between the per cent SRBC-R and the biochemical variables: vitamin A (r = 0.383), β-carotene (r= 0.879), vitamin E (r = 0.902) and zinc (r = 0.618) was evident.

b) Post partum

For the retention of placenta cases, there was a significant positive relationship (n=50, P<0.01) between per cent PBL-SRBC-R and the plasma biochemical variables vitamin A (r=0.914), β-carotene (r = 0.963), vitamin E (r = 0.964), and zinc (r = 0.949). In the control group buffaloes similar significant positive relationship (n=50, P < 0.01) of per cent PBL- SRBC-R with the plasma biochemical variables : vitamin A (r = 0.874), β-carotene (r=0.917), vitamin E (r = 0.940) and zinc (r= 0.900) were observed.

Table 2: Post partum per cent PBL - SRBC-R (Mean ± SEM) for normal and affected buffaloes

Days Post partum	Groups	
	Normal	Affected
1	7.10±0.32	4.15±1.03
2	7.20±0.61	4.15±0.38
3	7.45±0.49	4.05±0.26
4	6.95±0.08	4.95±0.29
5	16.95±0.43	6.30±0.34
10	44.40±0.11	26.25±0.63
15	52.40±0.18	32.50±0.50
20	56.75±0.31	35.00±0.37
25	55.35±0.25	45.05±0.29
30	55.55±0.36	52.00±0.25
CD between days (P<0.05)	1.13	1.59
CD between groups (P<0.01)	1.36	

Effect of plasma biochemical variables on PBL-SRBC-R

a) Pre partum and at the time of delivery

The overall existing effect of plasma biochemical variables on PBL- SRBC-R was 87.62 per cent (n=55) and 94.00 per cent (n=55) in control and affected groups, respectively.

The plasma vitamin A levels did not significantly effect the per cent PBL- SRBC-R in affected and control groups, whereas, in affected cases no significant effect of b-carotene on per cent PBL- SRBC-R was observed, however, in the control group animals the effect was significant (b= 0.303, P < 0.01). Similarly significant effect of vitamin E on per cent PBL- SRBC-R existed in the retention of placenta cases (b=0.479, P<0.01), as well as, control groups (b=0.365, P<0.01) whereas, zinc exhibited the significant inverse effect (b= -0.102, P<0.05) on per cent PBL-SRBC-R in the retained placenta cases only.

b) Post partum

Effect of plasma biochemical variables on per cent PBL-SRBC-R in the control group was existing (96.14 per cent, n = 50), whereas in the retained placenta cases it was 94.21 per cent (n = 50). Plasma β-carotene and vitamin E values affected significantly per cent PBL-SRBC-R in normally shed (b = 0.288, P < 0.05 and b = 0.265, P < 0.01, respectively) and retained placenta cases (b = 0.152, P < 0.05 and b = 0.169 and P < 0.01, respectively).

Table 3: Prepartum and calving plasma biochemical variables (mg/dl, Mean ± SEM) for normal and affected buffaloes

Period	Day	Vitamin A		β-carotene		Vitamin E		Zinc	
		Control	Affected	Control	Affected	Control	Affected	Control	Affected
Prepartum	10	18.95±1.79	19.95±1.33	89.72±0.52	91.12±1.03	184.01±1.75	186.58±0.61	155.88±6.38	154.41±2.94
	9	19.35±1.71	19.20±1.11	88.32±0.52	88.84±0.82	187.25±0.89	176.05±1.46	155.88±6.38	150.72±3.75
	8	18.72±1.73	18.72±1.13	87.48±0.68	87.94±0.79	191.43±0.68	181.56±3.17	155.88±5.66	147.05±2.95
	7	18.58±1.70	19.17±1.18	87.56±0.45	69.44±0.82	185.88±0.59	173.02±3.29	151.47±4.92	143.36±2.34
	6	18.55±1.50	18.15±0.92	86.04±1.39	66.42±0.64	179.23±1.05	162.13±2.87	152.94±3.84	139.71±4.65
	5	18.34±1.62	17.50±0.89	86.08±0.56	64.24±0.51	178.24±1.05	150.76±2.88	145.59±3.84	132.35±6.58
	4	16.22±1.46	17.25±0.83	65.60±1.37	62.66±0.51	177.60±1.31	139.09±2.90	145.59±3.84	128.68±3.75
	3	15.82±1.53	17.12±1.10	64.48±0.84	61.44±0.76	172.30±1.56	130.22±3.37	144.12±1.61	126.47±2.46
	2	16.70±1.53	16.98±1.12	64.56±1.01	61.56±0.93	158.12±0.43	131.82±0.98	144.12±9.67	124.53±2.73
	1	16.10±1.40	14.12±1.17	62.68±0.83	46.56±0.87	153.98±1.16	116.40±0.75	120.59±9.67	105.88±1.61
Calving	0	14.75±1.38	13.16±1.35	63.20±0.99	47.08±0.64	150.62±1.12	103.23±0.70	73.53±9.30	57.35±3.22
CD between days (P < 0.05)		NS	3.54	2.83	2.46	3.57	7.55	20.55	11.45
CD between groups (P < 0.05)		NS		2.62		5.82		NS	

Table 4: Postpartum plasma biochemical variables (mg/dl, Mean ± SEM) for normal and affected buffaloes

Days Postpartum	Vitamin A		β-carotene		Vitamin E		Zinc	
	Control	Affected	Control	Affected	Control	Affected	Control	Affected
1	13.24±1.14	12.53±1.28	58.28±1.45	47.16±0.61	154.80±1.17	100.14±2.65	52.94±5.66	41.18±4.46
2	13.06±1.03	12.24±1.09	57.20±1.37	46.64±0.65	156.68±0.84	100.20±2.80	45.59±5.26	44.12±3.60
3	12.44±1.00	12.10±1.28	52.28±1.33	44.52±0.95	171.12±3.20	102.04±2.80	51.47±5.88	50.00±6.38
4	12.39±0.95	10.90±0.95	55.88±1.44	43.76±0.49	173.12±1.59	110.34±1.66	61.76±4.92	52.94±3.84
5	12.31±0.77	11.56±0.95	58.84±1.45	45.96±0.61	179.54±1.08	119.44±1.44	73.53±9.30	61.76±4.92
10	22.84±0.76	12.43±1.25	101.88±1.45	47.56±0.43	229.72±1.64	218.14±1.44	138.24±6.03	64.70±6.71
15	26.01±0.68	20.91±0.42	111.08±4.62	104.20±0.60	229.44±1.76	228.14±1.11	148.53±3.84	125.00±8.80
20	25.56±0.66	23.14±0.80	118.92±2.20	108.04±0.39	225.70±2.22	233.75±0.39	158.82±6.10	155.88±5.66
25	25.78±0.64	24.08±0.94	119.36±0.90	111.92±1.29	232.40±0.64	230.37±0.20	158.82±6.10	155.88±6.38
30	25.10±1.46	25.60±0.80	119.48±1.48	114.48±1.06	232.32±0.65	229.62±0.51	158.82±6.10	152.94±3.84
CD between days (P < 0.05)	3.01	3.22	6.48	2.42	5.30	6.53	19.36	18.14
CD between groups (P < 0.05)	3.07		4.82		5.85		18.48	

Plasma zinc levels had no significant effect on the per cent PBL- SRBC-R count.

T-lymphocytes are known to have receptors (CD2) for SRBC, and this ability to bind heterologous red blood corpuscles helps in their differentiation from other classes of lymphocytes. The observations in the present investigation were similar to those found in cows by Yablonskii and Prigara (1984) who reported a significant reduction of T- and B-lymphocytes, 1-3 days before, and 3-5 days after calving in the normal and, to a higher degree, in the abnormal parturitions, the values were restored to the normal physiological levels 18-20 days after normal and 25-30 days after abnormal calvings. Lowered peripheral blood levels of T-lymphocytes have been reported in pregnant buffaloes (Arya, 1982), women (Bulmer and Hancock, 1977, Bolis *et al.*, 1979, Cornfield *et al.*, 1979). The reduction in T-cells may be associated with the maternal psychological stress (Crary *et al.*, 1983). Significant reduction in the number of lymphocytes present in the uterine epithelium during pregnancy have been reported (Wiel vander and King, 1984), but no characterization T-cells in the peripheral blood in relation parturition have been studied.

Based on, immunotrophism conc (Wegmann, 1990) the expulsion of placenta i

direct result of a response triggered by specific T-cell recognition of the allo-Major Histocompatibility Complex (MHC) antigens on the placental tissues, whereby the tissue injury is caused by the alloreactive effector cells. The actual detachement of the villi is for the greater part a mechanical process, which leads to tissue damage subsequently involving activation and migration of cells with phagocytic or non-specific lytic activity. Sufficient maturation of the placenta involving alterations in cellular activities is necessary for this loosening process (Grunert, 1986, Bjorkman and Dantzer, 1987).

Immune status is generally recognized as an indicator of health status, besides serum uric acid and serum cholesterol, which is modified by stress. Correlations of biochemical variables which have been demonstrated to be related to immune function along with regression analysis has been explored. The significant positive correlation of PBL-SRBC-R with vitamin A, β-carotene, vitamin E and zinc suggests that PBL-T may be related to these biochemical variables.

The development of an immunological response requires the active proliferation of a relatively small number of antigen sensitive lymphocytes to give a population of sensitized cells larger enough to be effective. Antigen-specific proliferative response of T-lymphocytes increases with dietary vitamin A intake from 0 to 6.6 mg/kg diet (Halevy *et al.*, 1994) in chicks suggesting influence of vitamin A status *in vivo* on the proliferative response of T-lymphocytes.

The effect of dietary vitamin A on different aspects of the immune response have been demonstrated by Friedman and Sklan (1993). Excess/deficiency of vitamin A causes impairment of immune response, as demonstrated by depression of antigen specific antibody production and T-lymphocytes proliferation in both animal (Friedman and Sklan, 1989 a & b, Pasatiempo *et al.*, 1991) and humans (Prabhala *et al.*, 1991). Replenishment of vitamin A in depleted or deficient subjects led to restoration of immune functions (Friedman and Sklan, 1989b, Friedman *et al.*, 1991). The molecular and cellular mechanisms that underly the interaction between vitamin A and immune response have not yet been elucidated.

Recent studies have demonstrated the role of retinoids as regulators for normal murine T-lymphocyte proliferation and activation (Garbe *et al.*, 1992, Friedman *et al.*, 1993). Optimal dietary supply of vitamin A is required for efficient T-lymphocyte-immune response (Halevy *et al.*, 1994). Significant positive correlation of nutrients related to immune response suggests that immune response may be related to protein anabolism/catabolism and therefore, of infant low body weight (Edwards *et al.*, 1994).

Serum nutrients have been associated with immune response in human subjects, zinc is well known to exhibit an immune modulating effect. Vitamin E is required for optimal response. Meydani *et al.* (1992) reported that higher levels of vitamin E than the recommended dietary allowances are needed to maintain optimal immune response in human subjects. Kowdley *et al.* (1992) observed reversible impairment in T-cell mediated immune function, in vitamin E deficiency, on vitamin E supplementation.

In the present study significant low T-lymphocyte profile in retention of placenta cases, might have led to retention, implying that lower levels were not able to mount a full response against the compatible antigens of the calf for regulating optimum placental growth and maturation. Thus, it is concluded that the peripheral blood profiles of T-lymphocytes periparturient may serve as an index of retention of fetal membranes. Extensive studies on the phenotypes of the cell population present in and around the placentomes at term, on lymphokine and cytokine production and manipulation thereof on cellular interactions at the fetal maternal contact sites are required to gain a better insight into the cellular basis of placental expulsion/retention.

The most consistent differences between the normally shed and retained placenta groups were in the vitamin A values. In these groups, at the pre partum and on day of calving, there was a significant mean difference in the per cent SRBC-R and other biochemical variables. For the retained placenta buffaloes, the positive significant relationship between pre partum and on day of calving PBL-SRBC-R and plasma vitamin A values are interesting findings. With regard to the relationships between these variables the two groups presented very different correlative results. It is possible that, for buffaloes with retained placentae, more than for buffaloes without retained placentae, initial PBL-SRBC-R and vitamin A values are among the important predictors of placental retention. To generalize from these findings, it may be concluded, that the better the initial state of health of the buffaloes, in terms of their PBL-SRBC-R status, the better the pregnancy

outcome. Also for vitamin A values during the third trimester and at calving, the data indicated that retention of placenta is associated with abnormally low levels of PBL.

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