EFFECT OF PRE-PARTUM SUPPLEMENTATION OF VITAMIN E TO MURRAH BUFFALOES ON LYMPHOCYTE FUNCTION OF CALVES

A.K. Singh*, S. Pandita, R.C. Upadhyay and G. Chandra

ABSTRACT

The present study was undertaken in buffalo neonates born to vitamin E supplemented (treatment group) and non-supplemented (control group) Murrah buffaloes. Blood samples were collected into sterile vacutainer tubes from calves at day 0 (before colostrum feeding), 7, 28, and 126-post birth. The lymphocytes were separated and cultured in RPMI 1640 medium with mitogen phytohemagglutinin-P (PHA-P) for 36h at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2). The lymphocyte responsiveness *in vitro* evaluated in response to different nitric oxide compounds *viz*. S-Nitroso-N-acetylpenicillamine (SNAP), N^{ω}-Nitro-L-arginine methyl ester (L-NAME) and L-N^G-Monomethyl-arginine (NMMA). The blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant between different groups. The precolostrum stimulation index was significantly low (P<0.05) in all the calves. The mitogen induced lymphocyte blastogenesis was not affected by low levels (1.0 mM) of SNAP but significantly inhibited (P<0.001) at 10 mM in both the groups. At 10 mM of L-NAME the blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant at 10 mM of L-NMMA. Nitric oxide (NO) production by lymphocytes from all calves increased (P<0.01) progressively with age. The lymphocytes supplemented with SNAP (1.0 and 10 mM) significantly (P<0.001) produce higher amounts of NO in comparison to non-supplemented lymphocytes. At a low level (0.2 mM) of L-NAME and L-NMMA, the change in the NO production between days was significant in both the groups. However, at a higher level (10 mM) of L-NAME and L-NMMA, the differences were not found to be significant between days and between the two groups. The study demonstrates that buffalo calves lymphocytes have an active nitric oxide synthase (NOS) system. The present study also indicates that the immune competence of buffalo calf lymphocytes is not affected by prepartum supplementation of vitamin E to buffaloes.

Keyword: Murrah buffaloes, buffalo calf, cell mediated immunity, lymphocytes, nitric oxide

INTRODUCTION

The neonatal period represents a critical stage in the development of physiological and immunological functions. Metabolic instability during this period makes the neonate particularly sensitive to perinatal diseases that result in high mortality (Dwyer, 2008). The neonate has an

Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal-132001 (Haryana), India, *E-mail: dranil02@gmail.com

immature immune system compared with that of an adult. The calf immune system is more susceptible to oxidative stress during the neonatal period due to a weak defense system against superoxide radicals (Inemani *et al.,* 1999). The calves are lymphopenic at birth (Outteridge and Duffy, 1981; Manak, 1986). The lymphocyte concentration gradually increases with age (Osburn *et al*., 1974; Clover and Zarkowen, 1980). Nagahata *et al*. (1991) found that antibody-producing activity of lymphocytes was lower in calves up to 3 weeks after birth. In addition, Tizard (2004) reported that antibody-producing activity in suckling calves is low at least up to 1 month after birth. Osburn *et al*. (1974) depicted a suppressed PHA-induced lymphocyte blastogenesis in the newborn as compared to bovine fetal lymphoid cells at 90 and 121 days of gestation (Osburn *et al*., 1974; Rensbaw *et al*., 1977). Clover and Zarkower (1980) reported that both PHA and poke weed mitogen (PWM)-induced blastogenic responses of peripheral blood lymphocytes from 6-hr old, colostrum fed calves were suppressed relative to those from 4-day old calves. Rajaraman *et al*. (1997) reported that peripheral blood mononuclear cells (PBMC) from 1-wk-old calves fed colostrum and milk were functionally hypo-responsive when compared to PBMC from adult cattle. Person *et al*. (1983) suggested that although the lymphocytes of neonate calves had the ability to respond to mitogens, individual variations are there in the lymphocyte reactivity. Compared with adult cattle, calves have a low percentage of E rosette-forming cells (Outteridge and Duffy, 1981) and their lymphocytes show less mitogen-induced proliferation (Manak, 1986). Yang and Shultz (1986) reported that lymphocytes from young piglets showed greater sensitivity to prednisolone than lymphocytes from 6-month old pigs. Other functional differences include the capacity of

PBMC from young calves to produce inducible NOS, a component of bactericidal mechanisms of phagocytic leukocytes and interferon-γ (IFN), a pivotal cytokine in cell-mediated immunity, reduced secretion of other cytokines (Nonnecke *et al*., 2003), and reduced neutrophil function (Dore *et al*., 1991; Higuchi *et al*., 1997) have also been reported after birth.

Vitamin E has been reported to be an immunopotentiator, which not only provides additional immunity to dairy animals when fed peripartum but also has been reported to enhance calf viability (Amer and Hashem, 2008). Therefore, in order to improve the immune status of neonates, it is imperative to supplement pregnant animals with vitamin E to increase its level in the colostrum and thus transfer it to calves. Induction of the lymphocyte proliferative response induced by antigen/mitogen *in vitro* has been shown to be representative of cellular immunocompetence. This measure can potentially be used as an indicator of an individual's ability to mount an immune response to a specific pathogen or immunomodulators. To date, no study describing the age-related changes in lymphocyte function in buffalo calves as influenced by ante-partum supplementation of vitamin E to buffaloes is available. Furthermore, study on the *in vitro* effect of nitric oxide on lymphocyte function in the developing buffalo calf is unavailable. Therefore, the purpose of this study was to investigate the age-related changes in mitogen-induced lymphocyte proliferation and the effects of nitric oxide (NO) on *in vitro* immune competence in terms of the lymphocyte proliferative response in buffalo calves.

MATERIALS AND METHODS

The experiment was approved by the Institutional Animal Ethics Committee constituted as per the article number 13 of the CPCSEA rules, laid down by the Government of India.

Selection of animals

The experiment was conducted on twenty apparently healthy Murrah buffaloes in an advance state of pregnancy selected from the National Dairy Research Institute (NDRI), Karnal herd, from October 2009 until May 2010. These were randomly divided into two groups (10 in each group). The control group was fed the diet as practiced for pregnant buffaloes on the NDRI dairy farm. The treatment group was supplemented with 2000 IU vitamin E (*dl-alpha-tocopherol acetate*) mixed with moistened concentrate and fed individually in the morning each day with the normal diet. Vitamin E supplementation was initiated 30 days prior to the expected date of parturition and was continued until parturition. Calves born from these buffaloes (both the groups) were removed from their dams before colostrum ingestion and housed in a calf's pen separately in two groups after weighing and ear tagging. The colostrum of the same dam was fed to the newborn within 2 h of birth, which was designated as 0 h, and subsequent feedings at an interval of 12 h for five days was followed.

Collection of blood and separation of lymphocytes

Approximately 15 ml of blood was drawn in sterile, heparinized vacutainer tubes (BD Franklin, USA) from each calf by jugular venipuncture on day 0 (before colostrums feeding), and on days 7, 28, and 126 post-birth at 6.00 AM in the morning.

Blood was centrifuged at 3000 rpm for 40 min at 4°C; the buffy coat was harvested and re-suspended in 1:1 v/v Dulbecco's Phosphate Buffer Saline (DPBS). The total contents were carefully layered on lymphocyte separation medium (Histopaque 1077) in a ratio of 4:1 v/v in a sterile 15 ml polypropylene centrifuge tube and centrifuged at 1500 rpm for 40 minutes at room temperature. The lymphocyte rich layer was removed and washed (1200-1500 rpm; 10 minutes) twice with DPBS. The washed lymphocyte were resuspended in RPMI 1640 medium (3-4 ml) containing 25 mM HEPES buffer (Gibco Laboratories, Grand Island, NY) that was supplemented with 10% FCS (Hyclone), 2 mM L-glutamine, antibiotics (100 IU/ml of penicillin G and 100 µg/ml of streptomycin), antimycotics (5 µg/ml of amphotericin B) (all from Sigma Chemical Co., St. Louis, MO, USA). Cells were counted on a hemocytometer and cell suspensions were diluted with RPMI to 1 x 10^6 cells/ml. Viability of cells (>95%) was determined by trypan blue dye exclusion.

Lymphocyte proliferation in response to PHA-P and Nitric oxide

The lymphocytes (1x10^6 live lymphocyte/well) were cultured in 96 well flat-bottomed tissue culture plates. The cells were allowed to proliferate with and without mitogen (PHA-P) to determine the difference between cell proliferations. To the PHA-P positive wells, 20 µl PHA-P (5 µg/ml) was added. The effect of nitric oxide on the lymphocyte proliferation response was studied by using nitric oxide compounds *viz.* S-Nitroso-N-acetylpenicillamine (S-NAP) - a nitric oxide donor (0.1, 1.0, 10.0 mM/well) and nitric oxide synthase inhibitors i.e. N$^\omega$-Nitro-L-arginine methyl ester (L-NAME) and L-N^G-monomethyl-arginine (L-NMMA) (0, 0.2, 1.0, 5.0 and 10.0 mM/well). In all the cases, final culture volume was 200µl/well. The blank wells consisted of 200 µl of culture

media only. The culture plates were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 36 h.

MTT Assay

The proliferative response of lymphocytes was estimated using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay according to the procedure given by Mosmann (1983). MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals, which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent dimethyl sulfoxide (DMSO) results in the liberation of the crystals. The number of surviving cells is directly proportional to the level of the formazan product created. The plate was read at 450 nm with Microscan MS-5608A plate reader.

Statistical Analysis

All analysis was done using a Systat 12 software package. Data from different experiments are presented as mean ± SE. Analysis of variance of the data was done using RBD factorial design. The responses have been compared with respect to unstimulated cells for different experiments involving the mitogen and different immunomodulators (S-NAP, L-NAME and L-NMMA). Significance was considered at P<0.05 or is mentioned otherwise.

RESULTS AND DISCUSSION

In the present study, cell-mediated immunity was assessed by measuring the proliferation of mitogen-stimulated lymphocytes and nitric oxide (NO) produced by lymphocytes in buffalo calves under different experiments. The lymphocyte proliferation response was expressed as a stimulation index for mitogen stimulated and mitogen plus immunomodulator stimulated as a relative stimulation index. The blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant between different groups. The precolostrum stimulation index was significantly low (P<0.05) in all the calves. The values registered a significant rise on day 7 post birth followed by a decline subsequently. The ANOVA revealed that antepartum supplementation of vitamin E had a significant effect on the stimulation index (P<0.05) of mitogen-induced lymphocyte blastogenesis (Figure 1).

Effect of nitric oxide donor (SNAP) on blastogenic response of lymphocytes

Effect of different concentrations of SNAP on the blastogenic response of lymphocytes *in vitro* following the culture of lymphocytes in the presence of 1.0 and 10 mM of SNAP, the mitogen induced lymphocyte blastogenesis was not affected by low levels (1.0 mM) of SNAP. It was significantly inhibited (P<0.001) at 10 mM in both the groups. At the both SNAP concentrations (1.0 and 10 mM) the lymphocyte stimulation index was not significant between the different groups (Table 1).

Effect of nitric oxide synthase (NOS) inhibitors (L-NAME and L-NMMA) on the blastogenic response of lymphocytes

The blastogenic response of cultured lymphocytes was significantly reduced in the

presence of 0.2 and 10 mM of L-NAME and L-NMMA in all the animals of both groups. At 10 mM of L-NAME the blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant at 10 mM of L-NMMA. When the two groups were compared with respect to blastogenic response, the differences were not found to be significant between the two groups (Table 1).

The lymphocyte proliferation assay is a measure of immune activation/stimulation. This assay helps evaluate the immunostimulatory/ immunosuppressive activity of a mitogen. Cell-mediated immunity requires a full repertoire of functional T cells. T cells, which derive from the bone marrow and fetal liver, mature in the thymus. In the blood circulation, they account 40 % to 80 % of the lymphocytes.

The results of the present study are in general agreement with Rajaraman *et al*. (1997) who reported that PBMC from 1-wk-old calves fed colostrum and milk were functionally hypo-responsive when compared to PBMC from adult cattle. These variations were also influenced by season and the age of the bovine (Soper *et al.*, 1978). In piglet Hoskinson *et al.* (1990) found highest spontaneous proliferation in the newborn piglet at wk 0.5 but it decreased (P<0.008) over 75% by wk 1. Proliferation tended to decrease, but more gradually, through wk 6. The present study indicated that the animals in different groups exhibited different blastogenic responses to different levels of NOS inhibitors. Lymphocyte stimulation is widely used to measure (i) immune competence by stimulation of lymphocytes with phytomitogens (Douglas, 1972; Weigel *et al*., 1992); (ii) histocompatability by mixed leukocyte culture (Thorsby, 1974) and (iii) exposure to infectious agents by stimulation of lymphocytes

with specific antigens (Daguiliard, 1972).

In the present study, functional capacity of lymphocytes was further evaluated by nitric oxide (NO) production by lymphocytes and their responsiveness to immunomodulators. Secretion of nitric oxide (NO), which is essential for effective cell-mediated immunity, was also influenced by treatment with values lowering significantly in response to antepartum supplementation of vitamin E. The functional capacity of lymphocytes was further evaluated by adding different immunomodulators in culture. NO production by lymphocytes from all calves increased (P<0.01) progressively with age. However, mitogen stimulated cells produced less NO in culture until day 126 as compared to unstimulated cells of all the calves (Figure 2).

Nitric oxide is a small inorganic paramagnetic free radical gas, which acts as a biological messenger molecule transmitting signals within and between cells, exerting a number of biological actions including vasodilation, neurotransmission, cytotoxicity and modulator of inflammation and immunity. Nitric oxide is produced by lymphocytes, macrophage, endothelial cells and neutrophils. Although production of NO by leukocytes that are stimulated by pathogens is an important component of the innate immune system, excess production of this molecule can be damaging to the tissues of the host. Nitric oxide (NO) production is a component of the innate immune system that has not been well studied in neonates. Nitric oxide acts as an intracellular signaling molecule or as a neurotransmitter when produced in low quantities. When produced in higher quantities for extended periods, NO is involved in the killing of microorganisms and tumor cells (Nathan, 1995) and in hematopoiesis (Ouaaz, 1995). Chronic production of NO in
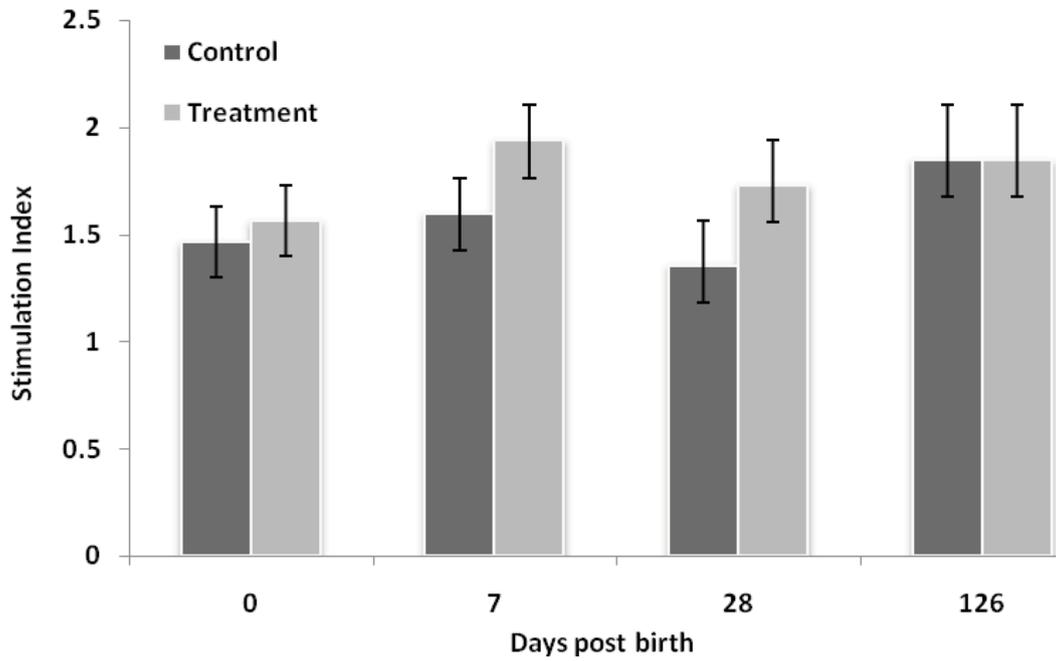
Figure 1. Age related *in vitro* changes in stimulation indices of buffalo calves in response to mitogen (PHA-P) stimulation.
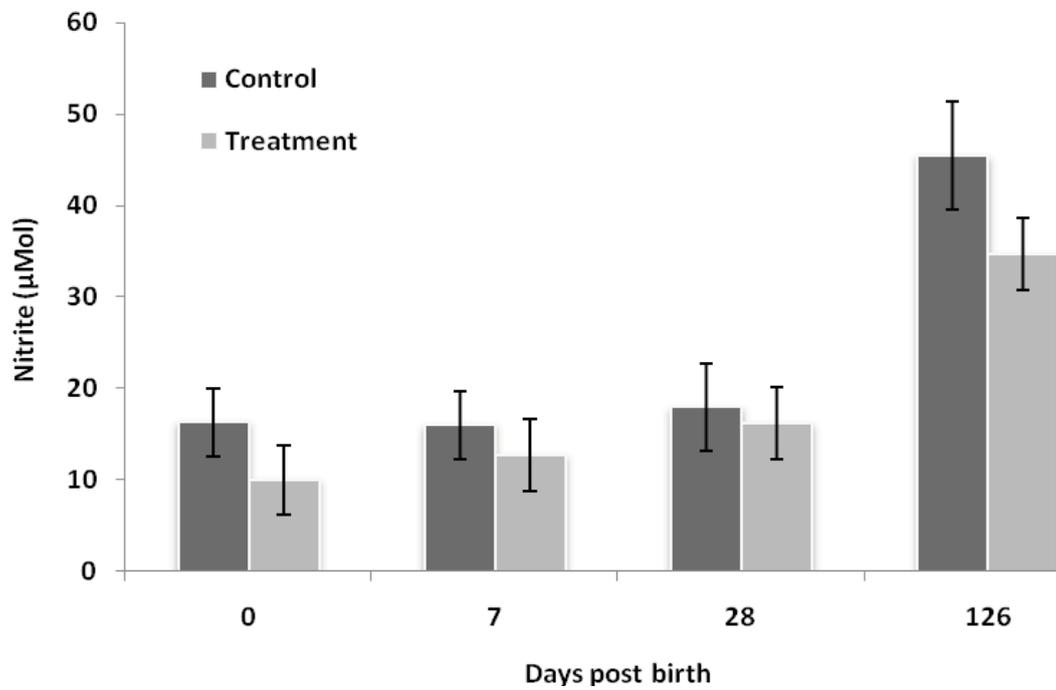

Figure 2. Age related changes in NO production of buffalo calves in response to mitogen (PHA-P) stimulation.

Table 1. Age related changes in relative stimulation Indices of buffalo calves in response to different immunomodulators.

Group	Days	SNAP		L-NAME		L-NMMA	
		1 mM/well	10 mM/well	0.2 mM/well	10 mM/well	0.2 mM/well	10 mM/well
Control	0	1.52±0.26	0.91±0.16	0.92±0.09	0.79±0.07	0.92±0.09	0.94±0.08
	7	1.32±0.26	0.30±0.16	0.67±0.09	0.53±0.07	0.93±0.09	0.82±0.08
	28	1.30±0.34	0.53±0.21	0.93±0.12	0.87±0.10	0.95±0.11	0.99±0.11
	126	0.90±0.42	0.15±0.26	0.64±0.14	1.01±0.12	0.91±0.14	1.07±0.13
Treatment	0	0.98±0.26	0.57±0.16	0.81±0.09	0.85±0.07	0.87±0.09	0.91±0.08
	7	1.34±0.28	0.47±0.17	0.96±0.10	0.76±0.08	1.15±0.09	1.11±0.09
	28	0.95±0.28	0.45±0.17	0.75±0.10	0.92±0.08	0.78±0.09	0.71±0.09
	126	0.89±0.28	0.16±0.17	0.78±0.10	0.98±0.08	0.95±0.09	1.01±0.09

association with superoxide anion generates toxic radicals, which can damage cell membranes, cause inflammation, and induce apoptosis (Dugas *et al.,* 1995).

Leukocytes from calves produced unusually high concentrations of NO when compared with those produced by cows, a possible indicator of the immaturity of the immune system of the neonatal calf. This reflected immature cell mediated immune functions in neonate buffaloes and could be explained on the basis of higher secretion of cytokines (interferon γ) by immune cells in the case of new born calves in response to any stress producing external stimuli (Nonnecke *et al.,*1993), thus exerting an inhibitory effect on NO production. Shoker *et al.* (1997) have also reported a relationship between IL-2 and NO in lymphocyte culture studies in humans. The higher the IL-2 production was, the lower the nitric oxide production. Rajaraman *et al.* (1998) showed that mitogen-stimulated PBMC from milk replacer fed calves produced more NO than parallel cultures of adult PBMC. They suggested that the age-related differences in NO secretion might be attributable to

the immaturity of the neonatal immune system.

Effect of nitric oxide doner (SNAP) on nitric oxide (NO) production by lymphocytes

Effect of different concentrations of SNAP on *in vitro* nitric oxide (NO) secretion by lymphocytes following culture of lymphocytes in the presence of 1.0 and 10 mM of SNAP were evaluated. The lymphocytes supplemented with SNAP (1.0 and 10 mM) significantly (P<0.001) produced higher amounts of nitric oxide (NO) in comparison to non-supplemented lymphocytes. In both the groups, nitric oxide (NO) secretion in culture supernatants was significantly (P<0.001) increased at both the concentrations (1.0 and 10 mM). However, the increase in the nitric oxide production at higher levels (10 mM) was much higher than at low levels (1.0 mM) of SNAP (Table 2).

Animal experiments suggest that the synthesis of nitric oxide by L-arginine-NO biosynthetic pathway affects immunoregulation (Moncada *et al.*, 1991). Hence, it was important to test in the first instance the maximum non-cytotoxic

Table 2. Age related changes in NO production of buffalo calves in response to mitogen and immunomodulators stimulation.

Group	Days	PHA-P	PHA-P + SNAP		PHA-P + L-NAME		PHA-P + L-NMMA	
		5 µg/ml	1 mM/well	10 mM/well	0.2 mM/well	10 mM/well	0.2 mM/well	10 mM/well
Control	0	16.33±3.73	49.78±6.19	377.46±25.17	15.77±2.85	13.86±2.93	15.71±3.09	14.64±1.69
	7	16.00±3.73	49.60±6.19	338.30±25.17	15.37±2.85	9.68±2.93	13.92±3.09	12.14±1.69
	28	17.95±4.81	55.15±7.99	470.09±32.49	17.32±3.67	10.12±3.78	12.89±3.99	9.72±2.18
	126	45.40±5.89	114.16±9.79	405.50±39.80	43.31±4.50	14.72±4.63	33.63±4.89	10.21±2.67
Treatment	0	9.98±3.73	50.60±6.19	351.29±25.17	9.82±2.85	8.49±2.93	7.17±3.09	8.64±1.69
	7	12.73±3.93	50.84±6.52	401.98±26.53	13.62±3.00	6.92±3.09	9.02±3.26	9.93±1.78
	28	23.19±3.93	47.26±6.52	367.40±26.53	22.19±3.00	9.26±3.09	13.86±3.26	9.86±1.78
	126	34.63±3.93	112.02±6.52	447.35±26.53	34.58±3.00	11.31±3.09	24.75±3.26	9.54±1.78

concentration of nitric oxide compounds. The maximum non-cytotoxic concentration of the nitric oxide donor (SNAP) using the trypan blue exclusion test was found to be 10 mM. At this concentration percent killing after exposing the lymphocytes for 24 h with SNAP was less than 5%. Shoker *et al*. (1997) using sodium nitroprusside (SNP) as a nitric oxide donor reported maximum inhibition at 2 mM concentration in human lymphocytes. SNAP decomposes at room temperature to produce nitric oxide. Nitric oxide in turn tends to affect the lymphocyte functions. Shoker *et al*. (1997) reported that human lymphocytes do not produce appreciable amounts of nitric oxide to affect lymphocyte mitogenesis. They further indicated that some of the nitric oxide generating compounds *viz*. sodium nitruprusside (SNP) and nitroglycerine (NG) had a potent but non-specific immuno inhibitory effect on human lymphocyte function by a mechanism other than nitric oxide production.

Effect of nitric oxide synthase (NOS) inhibitors (L-NAME and L-NMMA) on nitric oxide (NO) production by lymphocytes

The nitric oxide production in culture supernatants of lymphocyte was significantly inhibited in the presence of 0.2 and 10 mM of L-NAME and L-NMMA in all the animals of both groups. At 1.0 mM of L-NAME the nitric oxide production was not significant between different groups but at same concentration of L-NMMA, nitric oxide production was significantly ($P<0.05$) reduced in the treated group. At a low level (0.2 mM) of L-NAME and L-NMMA, the change in the nitric oxide production between days was significant in both the groups. However, at a higher level (10 mM) of L-NAME and L-NMMA, the differences were not found to be significant between days and between the two groups (Table 2).

L-NAME and L-NMMA are competitive inhibitors of the enzyme nitric oxide synthase (NOS). These work by preventing the availability of the substrate for NOS i.e. L-arginine to the enzyme resulting in reduced nitric oxide output. In the present experiment, the inhibitory effects of nitric oxide inhibitors (L-NAME, L-NMMA) at the lowest levels (0.2 mM) on lymphocyte proliferation further supported the contention that nitric oxide exerts a direct effect on lymphocyte mitogenesis.

CONCLUSIONS

In conclusion, these results indicate that the lymphocytes are responsive to nitric oxide compounds and produce significant amounts of nitric oxide to modify lymphocyte mitogenesis. Although production of NO by leukocytes that are stimulated by pathogens is an important component of the innate immune system, excess production of this molecule can be damaging to the tissues of the host. Production of high concentrations of nitric oxide is a possible indicator of the immaturity of the immune system of the buffalo calf. The immune competence of buffalo calf lymphocytes is not affected by vitamin E. Further studies are required to investigate the mechanisms and the pathways that affect mitogenesis in buffalo calves.

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