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Immune Response and Plasma Alpha Tocopherol and Selenium Status of Male Buffalo (*Bubalus bubalis*) Calves Supplemented with Vitamin E and Selenium

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ABSTRACT : An experiment was conducted using 20 male buffalo calves to study the effect of vitamin E and selenium supplementation on their immune response and plasma α-tocopherol and selenium status. These buffalo calves (10-12 months old, average body weight 75.30±2.20 kg) were randomly allotted to four treatments on the basis of their body weights and were fed on wheat straw and concentrate mixture to meet their nutrient requirements of 500 g/d body weight gain. The buffalo calves were fed either a control diet (neither supplemented with Se nor VE) or diets supplemented with Se at 0.3 ppm (+Se), DL-alpha tocopheryl acetate at 300 IU (+VE), and both DL-alpha tocopheryl acetate at 300 IU and Se at 0.3 ppm (+Se+VE). These experimental diets were fed for 180 days. Blood samples were collected at day 0 and subsequently at 45 day intervals up to 180 days of experimental feeding to monitor plasma α-tocopherol and Se concentrations. To assess humoral immune response, all calves were sensitized with formalin inactivated *Pasteurella multocida* antigen at 135 days of experimental feeding and blood was collected on 0, 7, 14, 21 and 28 days post vaccination (DPV) to measure antibody production using indirect ELISA. Cell mediated immune response of calves was assessed after 180 days of experimental feeding by *in vivo* delayed type hypersensitivity (DTH) reaction using phytohaemaglutinin-P (PHA-P) as a mitogen. Results revealed that feeding of VE and Se improved the plasma levels of these nutrients. Plasma levels of Se were affected by supplementation of both VE ($p<0.001$) and Se ($p<0.001$); however, no interaction (Se×VE) was observed. Supplementation of Se improved the humoral immune response ($p<0.008$), whereas, VE showed a tendency towards improvement in cell mediated immune response ($p<0.064$). It was concluded that vitamin E and Se supplementation improved the status of these micronutrients and humoral immune response in buffalo calves. (**Key Words :** Vitamin E, Selenium, Immune Response, Buffalo Calves)

INTRODUCTION

Vitamin E and selenium are micronutrients that share a common biological function in the animal body. Selenium is a component of glutathione peroxidase enzyme (Rotruck et al., 1973) which destroys free radicals in the cytoplasm, whereas vitamin E is a non-enzyme scavenger of free radicals (MacPherson, 1994) that functions as a specific lipid soluble antioxidant in cell membranes (Noguchi et al., 1973). Vitamin E reacts with peroxide radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide. This antioxidant activity of vitamin E in preventing lipid peroxidation may be one of the mechanisms by which vitamin E enhances immunity (Chew. 1996). Vitamin E causes a reduction of glucocorticoids. which are known to be immunosuppressive. It also alters arachidonic acid metabolism and subsequent synthesis of prostaglandin. thromboxanes and leukotrienes (Golub and Greshwin, 1985). Under stress conditions, increased levels of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function (Hadden. 1987). Similarly, selenium deficiency results in immunosuppression, reduced resistance to infection. neutrophil function, antibody production. proliferation of T and B cells in response to mitogens and cytodestruction by T lymphocytes and NK cells (Kiremidjian and Stotzky. 1987).

Samanta et al. (2006) in crossbred calves and Rajeesh (2006) in buffalo calves reported that supplementation of vitamin E improved the immune status of the animals.

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Mudgal (2006) found that supplementation of 0.3 ppm Se in buffalo calves significantly improved their humoral immune response, as compared to unsupplemented animals. Song et al. (2006) reported that supplementation of different levels of Se in the diet of laying hens significantly improved their lipopolysaccharide (LPS) stimulation index, concanavalin A (con A) stimulation index and peroxidase enzyme activity, while Lin and Chang, (2006) noticed enhanced immune response in cockrels when their diet was added with 20 mg vitamin E/kg of diet.

The National Research Council has recommended a dietary level of 0.3 ppm Se and 15-40 mg/kg DM of vitamin E for growing cattle (NRC, 1989). However, research suggests that relatively higher levels of vitamin E may improve calf performance (Secrist et al., 1997). As such, there appears to be no authentic report on the requirement of Se and vitamin E for buffaloes and often values recommended for cattle are used for this species. Hence, the present experiment was conducted to study the effect of supplementation of these two micronutrients on their plasma status and the immune response of male buffalo calves.

MATERIALS AND METHODS

Animals, their feeding and management

To carry out this experiment, twenty male buffalo (*Bubalus bubalis*) calves were procured from the local market. These animals were maintained on the experimental diet, comprising concentrate mixture and wheat straw, for two months during which they were treated against ecto- and endo-parasites before the start of the experiment and subsequently at regular intervals. All the calves were vaccinated against foot and mouth disease. These buffalo calves (n = 20; 10-12 months old, average body weight 75.30±2.20 kg) were distributed into four different groups; each of five animals on the basis of their body weights, following a complete randomized design. During the experimental period all the calves were kept in a well-ventilated shed with individual feeding and watering arrangements. The four groups were fed on wheat straw and concentrate mixture to meet nutrient requirements for a daily gain of 500 g/day (Kearl, 1982). The concentrate mixture consisted of 25% crushed maize grain, 30% soybean meal, 42% wheat bran, 2% mineral mixture and 1% common salt. Dietary treatments included: control diet (neither supplemented with Se nor VE) or diets supplemented with Se (from sodium selenite) at 0.3 ppm (+Se), DL-alpha tocopheryl acetate (Impextraco, Belgium) at 300 IU (+VE), and both Se at 0.3 ppm and DL-alpha tocopheryl acetate at 300 IU (+Se+VE). Available green fodder (2 kg) was given once a week to all the calves to meet their vitamin A requirement. Clean and fresh drinking water was provided twice a day to all animals.

Blood collection

About 10 ml of blood was collected in heparin from the jugular vein of each animal at the beginning and subsequently at 45 days intervals up to 180 days of experimental feeding. Blood was centrifuged at 700×g for 20 minutes for the separation of plasma, which was collected in sterile plastic vials and preserved in a deep freezer (-20°C) for biochemical analysis.

Estimation of vitamins in feed and plasma

Level of alpha tocopherol in concentrate mixture and wheat straw offered to the experimental calves was estimated by the method of McMurray et al. (1980), using High Performance Liquid Chromatography (HPLC). Retinol and alpha tocopherol concentrations in plasma were determined by the method of Milne and Botnen (1986) using HPLC. Standards of alpha tocopherol and retinol were procured from M/s Sigma Aldrich Chemicals,USA and were diluted using ethanol (Merck, Germany). Methanol (HPLC grade) was used as a mobile phase to maintain a flow rate of 2 ml/min. Detection of retinol was done at 305 nm and that of alpha tocopherol at 292 nm.

Estimation of selenium in feed and plasma

Selenium in feed and plasma samples was estimated by Atomic Absorbance Spectrophotometer (Model 4141, Electronic Corporation of India Limited, Hyderabad, India) using a nitrous oxide-acetylene flame, nitrogen as inert gas and sodium borohydrate (0.6% w/v in 0.5% NaOH) as a reducing agent. Samples of feed and plasma were digested using double acid (HNO_3, $HClO_4$; 4:1) mixture and volume was adjusted using double distilled water.

Humoral immune response

At 135 days of experimental feeding all calves were intramuscularly inoculated with a single dose (2 ml) of *Pasteurella multocida* oil adjuvant vaccine, and blood was collected at 0, 7, 14, 21 and 28 days post vaccination (DPV) to study antibody production for determination of humoral immune response. About 10 ml of blood was collected in clean-labeled tubes, centrifuged at 700×g for 20 minutes for separation of serum and harvested serum was carefully transferred to clean-labeled plastic vials and stored at -20°C for ELISA.

Preparation of experimental HS oil adjuvant vaccine

The experimental vaccine batch was prepared in the Division of Biological Products, IVRI, Izatnagar. *Pasteurella multocida* P_{52} (Type Culture Lab., IVRI) was grown in six Roux flasks containing approximately 125 ml Brain Heart Infusion agar (Hi-Media, India). After incubation for 18 h at 37°C, the growth from each flask was harvested in 10-15 ml of sterilized 0.15 M NaCl containing

0.5% (v/v) formalin using sterilized glass beads. Purity of the harvest from each bottle was checked under microscope in wet film using Gram staining. The pure harvests were pooled and kept at 37°C for 24 h. The pure pooled harvests of P52 cells in formal saline were filtered through a sterilized pad of muslin cloth and the filtrate was centrifuged at 2,000×g (Remi centrifuge, India) for 15 minutes. The pellet was washed three times in formal saline by centrifugation. Finally, the washed sediment was re-suspended in formal saline and matched with Brown's opacity tube no. 7. This suspension was finally mixed with oil adjuvant according to the method of Bain et al. (1982). Stability of the water in oil emulsion was checked by placing a drop of emulsion on a cold-water surface. The vaccine was stored at 4°C until used. Sterility and safety of the experimental vaccine was tested by the procedure laid down in schedule F_1 of the Drug and Cosmetics Act of Govt. of India (1994).

Preparation of sonicated antigen for ELISA

Roux flask containing Brain Heart Infusion (Hi- Media, India) was inoculated with pure nutrient broth culture of *Pasteurella multocida* P_{52} and incubated at 37°C for 18 h. The culture was harvested with normal saline solution (NSS) and sterilized glass beads. Purity of harvested culture was confirmed by Gram's staining technique and the contents were centrifuged at 2,000×g for 30 min. The sediments were washed twice with NSS and finally suspended in NSS to a density match with Brown's opacity tube no.7. The antigen was disrupted by 100-watt ultrasonic disintegrator at 6μ amplitude peak to peak for 30 minutes and used for ELISA (Almeida et al., 1979). The protein concentration was measured by the method of Lowry et al. (1951) and the concentration of protein used for ELISA was 10 μg/50 μl of the sonicated antigen.

Dilution of test sera samples

A final 1:50 dilution of serum sample was obtained by adding 5 μl serum to 245 μl of dilution buffer in each well of an uncoated protein binding 96 well micro titer plate. The diluted sera samples were allowed to equilibrate in dilution buffer for 5 min prior to transferring to antigen coated ELISA plate.

Preparation of controls

The positive (against *P. multocida*) and negative control sera of calves were diluted in dilution buffer to get a dilution of 1:100. Normal non-immunized calf serum was taken as negative control.

Test proper

For the test, the procedure laid down by Almeida et al. (1979) for ELISA was followed. Polystyrene plate (Griener, Germany) wells were covered individually with 50 μl of *P. multocida* (P52 strain) antigen containing approximately 10 μg protein. Then 50 μl of coating buffer (pH 9.6) was added to each well and the plates were incubated at 4°C in a refrigerator for 24 h. Excess buffer solution from the well was discarded by inversion and 50 μl of methyl alcohol was added to each well and incubated at 37°C for 2 h for drying. The antigen-coated plates were washed twice with washing solution. Fifty μl of serum dilution buffer was added to each well with the exception of the first three and last three wells of the first and sixth row. Fifty μl of unknown diluted (1:50) serum was added to each corresponding marked well of antigen coated test plate producing a final dilution of 1:100. The plate was then incubated at 37°C for 2 h. The liquid was tapped out from each well and then each well was washed with washing solution. A soaking time of 3 min was allowed and the procedure was repeated twice. Then 100 μl of suitably diluted conjugate (antibovine HRPO conjugate, procured from National Institute of Immunology, New Delhi, India) was dispensed into each assay well and the plate was incubated for 90 min at 37°C. Again the plate was washed twice with washing solution. Then 100 μl of substrate solution was pipetted into each test well and the plate was incubated at room temperature for 20 min. Later 100 μl of reaction stopping solution was added to each well. The ELISA plate was read with the ELISA reader (Electronic Corporation of India, Hyderabad, India) at 492 nm and then titer was calculated.

Cell mediated immune response

At the end of experimental feeding, an *in vivo* delayed type hypersensitivity (DTH) reaction against phytohemaglutinin-P (PHA-P) was carried out on all animals to determine the effect of vitamin E and Se supplementation on cell-mediated immune response. The skin on both sides of the neck was cleaned and shaved with a razor 24 h prior to injection, so that any inflammation set during the shaving or due to abrasion could subside. Then 150 μg of PHA-P in 200 μl of PBS (pH 7.4) was injected intra-dermally at two different sites 4 cm apart. The difference in skin fold thickness between PHA-P injected and control sites was measured with the help of Verniar caliper at 24, 48 and 72 h post sensitization.

Statistical analysis

The data generated was analyzed using SPSS (1996) by two-way ANOVA, with Se and VE as two factors and the effect of Se, VE and their interaction (VE×Se) were noted. The p-values compared the difference between Se, VE and their interaction.

Table 1. Chemical composition of feeds offered to buffalo calves (%DM basis)

Attribute	Concentrate mixture	Wheat straw
Organic matter	90.05	92.62
Crude protein	20.44	3.62
Ether extract	2.48	1.74
Acid insoluble ash	2.67	5.51
Neutral detergent fiber	41.48	82.33
Acid detergent fiber	13.19	56.73
Cellulose	9.53	48.58
Hemicellulose	28.28	25.61
Calcium	1.19	0.67
Phosphorus	0.96	0.07
Selenium (ppm)	0.17	0.13
Alpha-tocopherol (ppm)	13.15	2.00

RESULTS AND DISCUSSION

The chemical composition of wheat straw and concentrate mixture is presented in Table 1. The CP content of the concentrate mixture and wheat straw was 20.44 and 3.62 per cent respectively, whereas the alpha tocopherol and Se concentrations were 13.15 and 2.00 and 0.17 and 0.13 ppm, respectively.

The concentrations of alpha tocopherol, retinol and Se in the plasma of buffalo calves in different treatments are presented in Table 2. The plasma status of alpha tocopherol was affected ($p<0.001$) by supplementation of VE with +VE and +Se+VE treatments showing higher levels of alpha tocopherol, whereas Se had no effect on plasma alpha tocopherol. Similar to our findings, Walsh et al. (1993), Cusack et al. (2005) and Samanta et al. (2006) also reported that vitamin E supplementation in the diet of calves caused a significant increase in their plasma α-tocopherol concentrations. Supplementation of α-tocopherol in the diet of anestrous buffaloes steadily increased their serum tocopherol concentration (Kahlon and Singh, 2004); while VE supplementation from 60 days prepartum to 30 days postpartum had a beneficial effect on their plasma α-tocopherol level (Panda et al., 2006). A similar increase in the plasma α-tocopherol concentration of crossbred cows on supplementation with vitamin E (1,000 IU/cow/d) was reported by Chatterjee et al. (2003). Weiss et al. (1990) did not find any significant effect of Se supplementation (0.38 ppm) in Holstein cows on their plasma alpha tocopherol concentration, when compared to a control diet (0.11 ppm Se). Similarly, Mudgal (2005) did not observe any effect of supplemental Se (0.3 ppm) on plasma tocopherol concentrations in male buffalo calves. A vitamin E deficiency in cattle is characterized by a plasma concentration below 1.5 ppm (Hidiroglou et al., 1988); thus, none of our groups was deficient in this vitamin.

The concentration of retinol in the plasma of buffalo calves was not affected by the addition of vitamin E and/or Se in their diet. Contrary to our findings, Ezzo (1996) reported that Egyptian buffaloes supplemented with vitamin E and selenium had significantly ($p<0.05$) higher serum retinol concentrations as compared to unsupplemented buffaloes. However, similar to our results, Mudgal (2005) and Rajeesh (2006) reported that supplementation of Se and alpha tocopherol, respectively, in the diet of buffalo calves

Table 2. Selenium, alpha tocopherol and retinol levels in plasma of buffalo calves

Period (day)	Treatment[1]				SEM[2]	p-value[3]		
	Control	+Se	+VE	+Se+VE		Se	VE	Se×VE
Alpha tocopherol (ppm)								
0	1.76	1.71	1.70	1.61	0.16	0.932	0.001	0.272
45	1.78	2.25	4.11	4.09	0.37			
90	1.98	2.36	4.92	4.58	0.34			
135	2.88	2.91	5.38	5.29	0.30			
180	3.68	3.90	5.90	5.56	0.26			
Retinol (ppm)								
0	1.09	0.84	0.85	1.00	0.07	0.377	0.294	0.332
45	0.91	1.16	1.07	0.47	0.09			
90	0.77	1.02	0.96	0.84	0.09			
135	0.85	0.63	1.01	1.22	0.10			
180	1.25	1.25	1.74	1.39	0.09			
Selenium (ppb)								
0	148	146	136	154	6.21	0.001	0.001	0.214
45	162	202	174	237	8.61			
90	162	283	191	305	14.53			
135	174	320	194	352	18.13			
180	185	330	208	367	18.33			

[1] Control: without supplemental Se and VE; +Se: supplemented with 0.3 ppm Se;
+VE: supplemented with 300 IU vitamin E; +Se+VE: supplemented with both 0.3 ppm Se and 300 IU vitamin E.

[2] Standard error of the means.

[3] Se: effect of selenium; VE: effect of vitamin E; Se×VE: interaction between selenium and vitamin E.

Table 3. ELISA antibody titre of male buffalo calves vaccinated with *P. multocida* oil adjuvant vaccine

Period (day)	Treatment[1]				SEM[2]	p-value[3]		
	Control	+Se	+VE	+Se+VE		Se	VE	Se×VE
Antibody titre (days post vaccination)								
0	0.141	0.139	0.147	0.147	0.003	0.008	0.172	0.902
7	0.268	0.339	0.346	0.415	0.027			
14	0.355	0.437	0.385	0.488	0.029			
21	0.424	0.484	0.491	0.505	0.022			
28	0.432	0.481	0.375	0.476	0.023			

[1] Control: without supplemental Se and VE; +Se: supplemented with 0.3ppm Se;
+VE: supplemented with 300 IU vitamin E; +Se+VE: supplemented with both 0.3 ppm Se and 300 IU vitamin E.
[2] Standard error of the means.
[3] Se: effect of selenium; VE: effect of vitamin E; Se×VE: interaction between selenium and vitamin E.

Table 4. Skin fold thickness (cm) of male buffalo calves sensitized with PHA-P

Period (h)	Treatment[1]				SEM[2]	p-value[3]		
	Control	+Se	+VE	+Se+VE		Se	VE	Se×VE
0	0.60	0.57	0.61	0.69	0.04	0.206	0.064	0.870
24	0.90	1.02	1.00	1.06	0.05			
48	0.79	0.88	0.91	0.96	0.04			
72	0.68	0.74	0.80	0.78	0.03			

[1] Control: without supplemental Se and VE; +Se: supplemented with 0.3 ppm Se.
+VE: supplemented with 300 IU vitamin E; +Se+VE: supplemented with both 0.3 ppm Se and 300 IU vitamin E.
[2] Standard error of the means.
[3] Se: effect of selenium; VE: effect of vitamin E; Se×VE: interaction between selenium and vitamin E.

had no effect on blood retinol concentration.

The Se concentration in blood plasma of buffalo calves was affected by supplementation of both Se ($p<0.001$) and vitamin E ($p<0.001$); however, the interaction between Se and VE (Se×VE) was non-significant ($p>0.05$). Similarly, increased blood levels of Se due to Se supplementation were reported in cattle bulls (Pherson and Johnsson, 1985); Holstein cows (Weiss et al., 1990) and Hereford cows (Rowntree et al., 2004). Selenium levels below 20 ppb are indicative of a Se deficiency in cattle (Perry et al., 1976; Fenimore et al., 1983) suggesting that all the buffalo calves in this study had sufficient plasma Se levels.

The serum antibody titre against formalin inactivated *Pasteurella multocida* vaccine in buffalo calves at different days post inoculation (DPI), as measured by absorbance at 492 nm, is presented in Table 3. Supplementation of Se ($p<0.008$) improved the antibody titre in buffalo calves; however, vitamin E had no effect on the humoral immune response. Giadinis et al. (2000) reported that sheep injected twice with sodium selenite at 0.1 mg/kg body weight, at an interval of three weeks, had a significant ($p<0.05$) increase in Chlamydia antibody response. Beef cows that were given free choice access to salt mixes containing 20, 60 or 120 ppm Se as sodium selenite showed higher ($p<0.01$) concentration of serum IgG in the group having access to the high Se salt mix. Further, the calves born to cows given higher levels of Se (60 and 120 ppm) also had significantly ($p<0.05$) higher IgM and IgG concentration in their blood plasma compared to calves born to cows given a low level of selenium (Awadeh et al., 1998). Higher mean serum anti-Bovine Herpes Virus-1 antibody titers were observed in calves supplemented with 125 IU/d of vitamin E than the control calves and the response of calves supplemented with 500 and 250 IU/d was intermediate (Reddy et al., 1987). Similarly, Samanta et al. (2006) also reported higher levels of ELISA antibody titer against *Pasteurella multocida* antigen in calves supplemented with graded levels of vitamin E as compared to the control group. However, Muneer et al. (1994) reported no beneficial effect of feeding vitamin E on the humoral immune response against *P. multocida* immunization in buffalo calves.

The data pertaining to the skin fold thickness (SFT) of buffalo calves after sensitization with PHA-P is presented in Table 4. Supplementation of either vitamin E or selenium in the diet of buffalo calves had no effect on the cellular immune response. Similarly, Mudgal (2005) did not find any effect of supplemental Se (0.3 ppm) on the CMI response of buffalo calves. Contrary to our observations, Holstein heifer calves supplemented with vitamin E at 1,400 or 2,800 mg weekly had higher CMI responses (Reddy et al., 1985). Similarly, Ramos et al. (1998) reported that lambs supplemented with Se and vitamin E had greater responses to a delayed hypersensitivity test than control lambs. However, Beck et al. (2003) reported that calves from Se treated dams had a comparable CMI response in terms of skin swelling when compared to calves from control dams.

Thus, based on the results of this study it may be concluded that vitamin E and Se supplementation improved the status of these micronutrients in buffalo calves and also

improved their humoral immune response.

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