Effect of α-Tocopherol Supplementation on Plasma Levels of Antioxidant Vitamins in Anestrus Buffalo Heifers (*Bubalus bubalis*)

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ABSTRACT : The present investigation was undertaken to study the status of plasma antioxidant vitamins in normal cycling and α-tocopherol supplemented anestrus buffalo heifers. The pre-supplementation plasma levels (μmol/L) of vitamin E and β-carotene were significantly ($p<0.05$) lower and of vitamin C was significantly ($p<0.05$) higher in anestrus heifers (4.06±0.07; 4.56±0.17; 21.04±0.21) when compared to normal cycling ones (4.92±0.05; 6.76±0.12; 14.24±0.16). The oral supplementation of α-tocopherol at 3,000 mg per week per animal in anestrus heifers resulted in a significant ($p<0.01$) increase in vitamin E and β-carotene levels and a significant ($p<0.01$) decrease in vitamin C concentration. Results indicated that supplementation of α-tocopherol to anestrus buffalo heifers improved the antioxidant status by mitigating the harmful effects of free radical induced oxidative stress. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 8 : 1088-1092)***

Key Words : Antioxidant Vitamins, α-Tocopherol, Anestrus, Buffalo Heifers

INTRODUCTION

Under normal circumstances, oxidation and production of free radicals are an integral part of animal and human metabolism. Oxygen is the ultimate electron receptor in a closely linked electron flow system that produces energy in the form of ATP. However, when the electron flow becomes uncoupled, it leads to production of free radicals (Papas, 1996). Cellular defense against these free radicals provides both enzymatic (Kahlon and Singh, 2003) and non-enzymatic mechanisms. These mechanisms operate in concert and they target the removal of radicals or radical-initiated secondary products (Chan, 1993). Vitamins E, C and β-carotene are essential dietary nutrients that can scavenge free radicals and constitute a strong line of defense in retarding free radical induced cellular damage.

Vitamin E has been termed as necessary for proper functioning of various reproductive processes in the mammalian females (Ezzo, 1995; Jukola et al., 1996). The most widely accepted biological function of vitamin E is its antioxidant property. It is most effective chain-breaking lipid soluble antioxidant in the biological membranes, where it contributes to membrane stability. It protects critical cellular structures against damage from oxygen free radicals and reactive products of lipid peroxidation (Machlin, 1991; Rock et al., 1996).

Keeping in view the immense significance of vitamin E in removal of oxygen radicals and termination of free radical chain reactions, the present investigation was undertaken to monitor the status of antioxidant vitamins in normal cycling and α-tocopherol supplemented anestrus buffalo heifers.

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MATERIALS AND METHODS

Experimental animals

The investigation was conducted on 13 clinically healthy Murrah buffalo heifers between two to four years old and having more than 250 kg body weight. These animals were maintained as per standard feeding and managemental conditions practiced at the dairy farm of Punjab Agricultural University, Ludhiana, India (Latitude 30°45'; Longitude 75°48'). Buffalo heifers were selected on the basis of their reproductive history and status of reproductive organs as assessed by rectal examination before commencement of study.

Selection of antioxidant

α-Tocopherol was selected for supplementation because it is a non-toxic antioxidant and its toxicity has not been reported so far.

Grouping of animals

The buffalo heifers after selection were divided into two groups.

Anestrus group : Eight buffalo heifers with inactive and smooth ovaries and showing sexual quiescence for at least three preceding reproductive cycles were selected in this group. Five animals were supplemented orally with 3,000 mg α-tocopherol (as acetate) per animal per week for 12 weeks and remaining three animals were kept as control.

Normal cycling group : Five buffalo heifers showing

Table 1. Plasma antioxidant vitamins levels in normal cycling and α-tocopherol supplemented anoestrus buffalo heifers (Mean±SE)

Groups	Weeks of supplementation							Weeks of post supplementation	
	0	2	4	6	8	10	12	2	4
Vitamin E (μmol/l)									
Anoestrus treated	4.06^{a}±0.07	4.63^{a}**±0.05	5.18^{ac}**±0.07	6.17^{a}**±0.10	5.92^{a}**±0.08	5.97^{a}**±0.10	5.81^{a}**±0.10	5.58^{a}**±0.08	5.39^{a}**±0.08
Anoestrus control	4.14^{a}±0.12	4.29^{b}±0.04	4.25^{b}±0.06	4.25^{b}±0.08	4.31^{b}±0.09	4.29^{b}±0.04	4.32^{b}±0.08	4.35^{b}±0.09	4.35^{b}±0.08
Normal cycling	4.92^{b}±0.05	4.94^{c}±0.04	4.93^{c}±0.06	4.95^{c}±0.04	4.93^{c}±0.06	5.00^{c}±0.04	4.97^{c}±0.03	4.94^{c}±0.04	4.92^{c}±0.04
Vitamin C (μmol/l)									
Anoestrus treated	21.04^{a}±0.21	19.32^{ab}±0.29	17.85^{a}**±0.22	16.98^{a}**±0.32	17.33^{a}**±0.46	17.18^{a}**±0.63	17.39^{a}**±0.64	17.47^{a}**±0.52	17.74^{a}**±0.52
Anoestrus control	21.04^{a}±0.38	21.27^{a}±0.33	20.79^{b}±0.19	20.03^{b}±0.49	20.39^{b}±0.65	20.06^{b}±0.38	18.97^{ab}±0.33	21.23^{b}±0.19	20.98^{b}±0.51
Normal cycling	14.24^{b}±0.16	14.76^{c}±0.28	15.07^{c}±0.46	14.70^{c}±0.49	14.73^{c}±0.57	14.34^{c}±0.48	14.37^{c}±0.53	14.37^{c}±0.36	14.16^{c}±0.43
β-Carotene (μmol/l)									
Anoesrtus treated	4.56^{a}±0.17	5.48^{a}**±0.11	5.56^{a}**±0.13	5.84^{a}**±0.14	6.18^{a}**±0.08	6.05^{a}**±0.13	6.25^{a}**±0.08	5.49^{ac}**±0.13	5.32^{ab}**±0.13
Anoestrus control	4.59^{a}±0.27	4.56^{b}±0.27	4.61^{b}±0.32	4.62^{b}±0.25	4.58^{b}±0.26	4.61^{b}±0.20	4.60^{b}±0.25	4.64^{b}±0.25	4.67^{a}±0.26
Normal cycling	6.76^{b}±0.12	6.78^{d}±0.13	6.80^{c}±0.13	6.80^{c}±0.12	6.80^{c}±0.13	6.73^{c}±0.14	6.67^{a}±0.14	6.70^{d}±0.13	6.66^{c}±0.10

The values having same superscripts within a parameter column don't differ significantly ($p<0.05$) from each other.
The values having asterisk (** $p<0.001$, * $p<0.05$) within a row differ significantly from pre supplementation value.

normal estrus cyclicity during two preceding estrus cycles were selected in this group.

Sampling schedule

The blood samples of anestrus and normal cycling buffalo heifers were collected at weekly interval and data of four samples was pooled to establish pre-supplementation base line.

The blood samples were collected at fortnightly interval for 12 weeks in normal cycling and α-tocopherol supplemented anestrus heifers.

The blood samples were collected in both groups at fortnightly interval for four weeks during post-supplementation period.

Blood samples were collected aseptically from jugular vein in heparinized glass stopper vials. Plasma was separated by a refrigerated centrifuge at 3,000 rpm for 15 minutes and stored at -20°C in different aliquots for the analysis of plasma antioxidant vitamins.

Biological procedures

The vitamin E was estimated by method of Kayden et al. (1973) and is based on the principle that vitamin E reduced ferric ions to ferrous ions quantitatively, which combined with bathophenanthroline to form an orange coloured complex. After adding phosphoric acid to stabilize complex, the colour was read at 536 nm.

The vitamin C was estimated by 2,4-dinitrophenylhydrazine (DNPH) method as described by Baker and Frank (1968). It is based upon principle that coupling of DNPH to the keto groups of carbon 2 and 3 of diketogulonic acid yielded osazone called bis-2, 4-dinitrophenylhydrazone. In strong acid, this osazone rearranged to a stable reddish-brown product, which was measured photometrically at 505 nm. In this method, 2,6-dichlorophenolindophenol oxidized ascorbate to dehydroascorbate which, in strong acidic medium, hydrolyzed to diketogulonic acid so that hydrazone formation could take place.

The β-carotene was assayed by method of Baker and Frank (1968). It is based upon principle that the precipitation of proteins with ethanol and extraction of β-carotene into light petroleum was followed by reading of intensity of yellow colour due to carotene at 450 nm.

Statistical analysis

Data was subjected to one way analysis of variance (ANOVA) on computer using GraphPad InStat Programme developed by Peter Russell, Royal Veterinary College London 9508375. Regression analyses were carried out using Microsoft Excel programme.

RESULTS AND DISCUSSION

The plasma concentrations of vitamins E, C and β-carotene in normal cycling and α-tocopherol supplemented

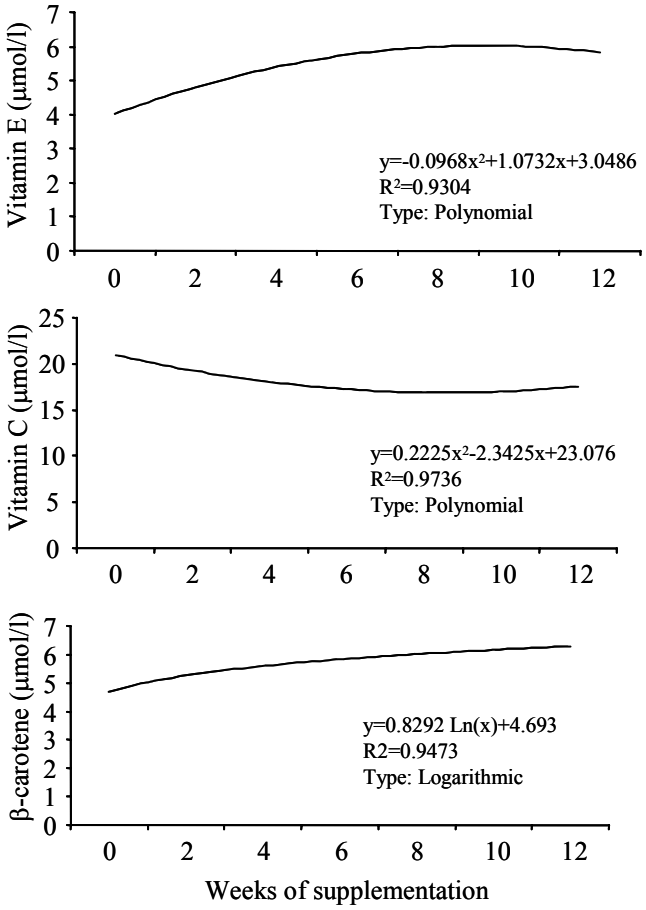

Figure 1. Regression analysis of plasma vitamin E, vitamin C and β–carotene levels in α–tocopherol supplemented anoestrus buffalo heifers.

anestrus buffalo heifers are presented in Table 1.

Plasma vitamin E

The level of plasma vitamin E (µmol/L) before supplementation in anoestrus (4.06±0.07) heifers was significantly ($p<0.05$) lower as compared to normal cycling group (4.92±0.05). The plasma level of vitamin E increased significantly ($p<0.01$) and reached at peak level by 6th week of α-tocopherol supplementation in anoestrus animals (Table 1). A polynomial regression of second order between plasma vitamin E levels and weeks of α-tocopherol supplementation was significant ($p<0.05$) in anoestrus ($R^2=0.9304$) buffalo heifers (Figure 1).

In anoestrus and normal cycling mares, the levels of plasma vitamin E were 0.302 and 0.597 mg/dl respectively (Mert et al., 1992). They were of the view that deficiency of vitamin E could be the cause of the anoestrus. Supplementation of feed with vitamin E increased serum α-tocopherol concentration linearly in gilts and animals fed on high vitamin E diet (113.5 mg/kg) had 16% lower anoestrus than those fed on low vitamin E diet (23.5 mg/kg) (Grandhi et al., 1993). Similarly, Jukola et al. (1996) reported lower serum vitamin E levels in anoestrus than normal cycling cows (4.0 vs. 5.9 mg/l). The oral supplementation of α-tocopherol at 3,000 mg per week per animal in anestrus heifers declined erythrocytic superoxide dismutase and glucose-6-phosphate dehydrogenase activities significantly ($p<0.01$) but led to non-significant increase in erythrocytic glutathione peroxidase activity (Kahlon and Singh, 2003).

The main function of vitamin E in the body is to act as a lipid soluble antioxidant, protecting cells against oxidative destruction. Vitamin E is a potent chain-breaking antioxidant, scavenging oxygen radicals and terminating free radical chain reactions (Rock et al., 1996). After interaction with the free radical, the resultant α-tocopheroxyl radical can be either regenerated back to α-tocopherol by ubiquinol, reduced glutathione and vitamin C (Machlin and Bendich, 1987; Chan, 1993) or oxidized by peroxyl radicals to inactive products (Liebler and Burr, 1995).

The low levels of plasma vitamin E in anoestrus buffalo heifers (Table 1) indicated oxidative stress in these animals. During quenching of free radicals, vitamin E is depleted from the body thus decreasing its plasma level. The supplementation of α-tocopherol in anoestrus heifers improved their antioxidant status as is evident from increased level of plasma vitamin E, decreased lipid peroxidation and osmotic fragility of erythrocytes (Kahlon, 1998; Kahlon et al., 2002). The role played by vitamin E in fertility is still disputed in cattle and buffalo. The fact that the endocrine glands, particularly the pituitary, have high vitamin E levels in comparison with other organs, would support the hypothesis that vitamin E affects reproduction (Garnsworthy, 1988). Moreover, vitamin E is thought to promote release of FSH and LH from adenohypophysis (Barnes and Smith, 1975). The deficiency of vitamin E can negate the fertility indirectly through liver disorders, which in turn are related to fertility disturbances (Lotthammer, 1975). In addition vitamin E can protect the conjugated double bonds of β-carotene and vitamin A (Machlin and Bendich, 1987) that are essential for fertility in cattle and buffalo.

Plasma vitamin C

The pre-supplementation plasma vitamin C level (µmol/l) in anestrus heifers (21.04±0.21) was significantly ($p<0.05$) higher than normal cycling heifers (14.24±0.16) (Table 1). The plasma level of vitamin C decreased significantly ($p<0.01$) to attain nadir levels at 6th week of supplementation in anoestrus buffalo heifers. The regression analysis revealed a polynomial relationship of second order between weeks of supplementation and levels of vitamin C in supplemented anoestrus buffalo heifers ($R_2=0.9736$; Figure 1). There was a highly significant ($p<0.01$) inverse

correlation between plasma vitamin C level and plasma level of vitamin E (r=-0.9620) and β-carotene (r=-0.9233) in anoestrus buffalo heifers.

Unlike human and other primates, the domestic animals including buffalo can synthesize ascorbic acid from D-glucose because of the presence of L-gulonolactone oxidase enzyme, which converts L-gulonolactone to 3-keto-L-gulonolactone. The increased plasma vitamin C levels during anoestrus (Table 1) could be due to compensatory induction of *de novo* synthesis of vitamin C in an attempt to combat oxidative and peroxidative challenge. Moreover, high concentration of vitamin C is a prerequisite for regeneration of vitamin E from tocopheroxyl radical (Draper, 1990). The high plasma vitamin C levels in the heifers might be a physiologic adjustment in order to keep the vitamin E in active form. During supplementation of α-tocopherol, level of plasma vitamin C tend to decline subsequent to sparing effect of vitamin E on vitamin C as well as inhibition of its endogenous synthesis.

Plasma β-carotene

The plasma β-carotene level before supplementation in anoestrus animals was significantly ($p<0.05$) lower than normal cycling group (Table 1). The levels of plasma β-carotene increased significantly ($p<0.01$) in α-tocopherol supplemented anoestrus buffalo heifers and reached to peak level of 6.18±0.08 μmol/l at 8th week of supplementation. The regression analysis revealed a logarithmic relationship between weeks of supplementation and plasma β-carotene levels in supplemented anoestrus buffalo heifers (R_2=0.9473; Figure 1). A significant ($p<0.05$) direct correlation between plasma levels of β-carotene and vitamin E (r=0.8917) and a significant ($p<0.01$) negative correlation between plasma levels of β-carotene and vitamin C (r= -0.9233) were observed in α-tocopherol supplemented anoestrus buffalo heifers.

Mert et al. (1992) reported normal plasma β-carotene level of 27.94 μg/dl in cycling mares and decreased level of 24.11 μg/dl during anoestrus condition. Supplementation of vitamin E and fat increased the concentration of plasma β-carotene (Weiss et al., 1994).

The major biological function of β-carotene is its potential role as vitamin A precursor. Lotthammer et al. (1978) suggested that β-carotene has a vital role in reproduction in cows that is unrelated to vitamin A. Goto et al. (1989) found that superovulated cows with high plasma β-carotene concentration had higher embryonic survival rate than those with low concentration. However, other workers found no effect of β-carotene on plasma progesterone, corpora lutea size and frequency or amplitude of LH pulses (Wang et al., 1988)

β-Carotene is an efficient quencher of singlet oxygen and can directly scavenge free radicals (Krinsky, 1993; Stahl and Sies, 1993). It can also function as a chain breaking antioxidant in the lipid phase by neutralizing peroxyl radicals (Larson, 1997). β-Carotene is an unusual antioxidant in the sense that it exhibits good radical trapping antioxidant behavior but only when partial pressure of oxygen is significantly less than 150 torr, the pressure of oxygen in normal air (Burton and Ingold, 1984). This does not exclude an important biological antioxidant role of β-carotene since partial pressure of oxygen in most of tissues under physiological conditions found low.

During anestrus, the oxidative stress tended to persist as was evident from the decreased plasma vitamin E level and increased lipid peroxidation and osmotic fragility of erythrocytes (Kahlon, 1998; Kahlon et al., 2002). The oxidative stress caused depletion of β-carotene from body. Supplementation of α-tocopherol has also sparing action on β-carotene by protecting its double bonds from oxidation. Moreover, positive influence of vitamin E on plasma β-carotene concentration is because of its increased transport capacity, increased intestinal absorption and increased mobilization of stored β-carotene especially from adipose tissues.

CONCLUSION

The study revealed that the decreased plasma levels of vitamin E & β-carotene observed in anoestrus buffalo heifers implied to occurrence of oxidative stress and poor antioxidant status in these animals. The increased plasma vitamin C levels suggested an adaptive response of anoestrus heifers to oxidative stress in an attempt to improve the antioxidant status. The supplementation of α-tocopherol to anoestrus buffalo heifers mitigated the effects of oxidative stress to improve antioxidant status as elucidated by increase in plasma levels of vitamin E and β-carotene and decrease in plasma level of vitamin C. Two anoestrus buffalo heifers exhibited estrus cyclicity during experimental period while one anoestrus heifers came into estrus only after completion of the experiment. However, the remaining two anoestrus buffalo heifers never showed signs of estrus during and after the experiment. The results of the present communication are, therefore, of paramount importance for laying the foundation of physiological norms of antioxidant vitamins in normal cycling and anoestrus buffalo heifers and would be a useful index for further research in the field of reproductive and nutritional physiology of buffaloes.

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