Status of Antioxidant Enzymes in Normal Cycling and α-Tocopherol Supplemented Anestrus Buffalo Heifers (*Bubalus bubalis*)

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ABSTRACT : The present investigation was undertaken to study status of erythrocytic antioxidant enzymes in normal cycling and α-tocopherol supplemented anestrus buffalo heifers. The pre-supplementation erythrocytic activities of superoxide dismutase (U/mg Hb), glutathione peroxidase (U/mg Hb) and glucose-6-phosphate dehydrogenase (U/g Hb) upregulated significantly ($p < 0.05$) in anestrus heifers (10.08 ± 0.09, 14.09 ± 0.54, 9.25 ± 0.29) when compared to normal cycling ones (6.93 ± 0.04, 11.61 ± 0.19, 5.58 ± 0.26). The oral supplementation of α-tocopherol @ 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. Results indicated that supplementation of α-tocopherol to anestrus buffalo heifers mitigated the effects of oxidative stress to improve their antioxidant status. (*Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 2 : 217-221*)

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

INTRODUCTION

Oxidative stress, caused by increased formation of free radicals and reactive oxygen species (ROS) through different metabolic processes and in response to exogenous stimuli, is controlled by various antioxidant defense mechanisms including antioxidant enzymes (Rock et al., 1996). Superoxide dismutase (EC 1.15.1.1; SOD), glutathione peroxidase (EC 1.11.1.9; GPX) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G6PD) prevent the accumulation of free radicals and ROS by inhibiting their formation (Zoeren-Grobben et al., 1994). Vitamin E is a potent chain breaking antioxidant, which inhibits propagation of peroxidation reactions by scavenging oxygen radicals and terminating free radical chain reaction (Burton and Traber, 1990; Ferrell and Robert, 1994).

Anestrus is a serious problem responsible for reproductive inefficiency of buffaloes. In the past, considerable research has been conducted in the field of reproductive endocrinology to identify specific problems of reproduction and adopt measures to improve reproductive efficiency of buffalo. However, little information is available in literature regarding antioxidant defense mechanisms during anestrus in buffalo. Therefore, the present communication reports erythrocytic activities of antioxidant enzymes in normal cycling and α-tocopherol supplemented anestrus buffalo heifers.

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.

i) 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. The amount of α-tocopherol supplementation was based on requirements of vitamin E as described by Putnam and Comben (1987).

ii) Normal cycling group: Five buffalo heifers, showing

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

Sampling schedule:

i) 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.

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

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

Sampling

Blood samples were collected aseptically from jugular vein in heparinized glass stopper vials and processed for preparation of hemolysate. After preparation, hemolysates were quickly stored at -20°C and enzyme activities were estimated within two days.

Biological procedures

SOD activity was measured by inhibiting the reduction of nitroblue tetrazolium (NBT) with reduced nicotinamide adenine dinucleotide (NADH) mediated by phenazine methosulfate (PMS) under aerobic conditions at 560 nm (Nishikimi et al., 1972). GPX activity was assayed following oxidation of reduced glutathione (GSH) in the presence of H_2O_2 at 412 nm (Hafeman et al., 1974). G6PD activity was measured following reduction of $NADP^+$ at 340 nm in the presence of glucose-6-phosphate (Deutsch, 1978). The vitamin E was estimated by method of Kayden et al. (1973).

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 α-tocopherol concentrations and erythrocytic activities of SOD, GPX and G6PD in normal cycling and α-tocopherol supplemented anestrus buffalo heifers are presented in Table 1.

Superoxide dismutase

The pre-supplementation erythrocytic SOD activity in anestrus heifers was significantly ($p < 0.05$) higher than those in cyclic animals. Supplementation of α-tocopherol,

however, decreased erythrocytic SOD activities significantly ($p < 0.01$) in anestrus heifers, which reached nadir levels at 10th week of supplementation, but the enzyme activity still remained higher than the corresponding activities in normal cycling heifers. The regression analysis revealed a significant ($p < 0.05$) polynomial relationship ($R^2 = 0.9786$) between weeks of supplementation and erythrocytic SOD activities in supplemented anestrus heifers (Figure 1).

SOD is a copper and zinc containing primary antioxidant enzyme that dismutates two-superoxide radicals ($O_2^{\bullet-}$) to H_2O_2 and O_2 (Figure 2). Increased lipid peroxidation and osmotic fragility of erythrocytic and decreased levels of plasma vitamin E and β-carotene in anestrus buffalo heifers implied occurrence of oxidative stress and poor antioxidant status in these animals (Kahlon, 1999). Therefore, the increased activities of erythrocytic SOD in anestrus heifers could be attributed to physiological upregulation of this enzyme in an attempt to mitigate superoxide radical challenge. Supplementation of α-tocopherol might be responsible for relieving the load of oxidative stress, thus lowering erythrocytic SOD activities in supplemented heifers.

Glutathione peroxidase

The erythrocytic GPX activities in anestrus heifers were significantly ($p < 0.05$) higher than normal cycling animals. During supplementation of α-tocopherol in anestrus subjects, the increase observed in erythrocytic GPX activity was statistically non-significant. A significant ($p < 0.05$) polynomial regression ($R^2 = 0.6437$) was seen between weeks of supplementation and erythrocytic GPX in supplemented buffalo heifers (Figure 1).

GPX is also primary antioxidant metalloenzyme containing four atoms of selenium per molecule of enzyme and catalyzes reduction of H_2O_2 and organic peroxides (ROOH) to their respective alcohol and water (Rotruck, 1973; Figure 2). The association between increased activity of blood GPX and incidence of anestrus or subestrus was reported by Jukola et al in a study on fertility disorders of cows (Jukola et al., 1996). Oxidative stress in anestrus buffalo heifers accounted for accumulation of H_2O_2 and ROOH as evident from increased erythrocytic SOD activity (Table 1) and malonyl dialdehyde levels (Kahlon, 1999). Therefore, elevated erythrocytic GPX activities during anestrus could represent upregulation of this enzyme for effective removal of H_2O_2 and ROOH. Vitamin E has been reported to reduce requirement of selenium by preventing loss of selenium from body or maintaining it in active form (Mayes, 1996). Hence, the non-significant increase in erythrocytic GPX activities during α-tocopherol supplementation in anestrus buffalo heifers could be the sparing effect of vitamin E on selenium.

Table 1. Plasma vitamin E levels and erythrocytic superoxide dismutase, glutathione peroxidase and glucose-6-phosphate dehydrogenase activities 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	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
SOD (U/mg Hb)									
Anoestrus	10.08[a]±0.09	9.40[a**]±0.05	8.59[a**]±0.05	8.26[a**]±0.04	8.30[a**]±0.04	8.24[a**]±0.04	8.29[a**]±0.05	8.33[a**]±0.02	8.40[a**]±0.04
Anoestrus control	10.20[a]±0.06	10.25[b]±0.06	10.15[b]±0.06	10.21[b]±0.10	10.18[c]±0.06	10.45[b]±0.10	10.17[b]±0.10	10.16[b]±0.08	10.10[b]±0.04
Normal cycling	6.93[b]±0.04	6.96[c]±0.04	6.96[c]±0.04	6.98[c]±0.05	7.01[c]±0.04	7.07[d]±0.03	7.06[c]±0.04	7.01[c]±0.05	6.93[c]±0.04
GPX (U/mg Hb)									
Anoesrtus	14.09[a]±0.54	13.88[a]±0.48	14.42[ab]±0.35	15.13[ab]±0.55	15.38[ab]±0.51	14.95[a]±0.46	14.20[a]±0.52	14.46[a]±0.48	14.14[a]±0.39
Anoestrus control	13.47[ab]±0.61	14.06[a]±0.14	13.09[a]±0.32	12.75[ac]±0.16	12.92[c]±0.29	13.07[b]±0.26	13.03[ab]±0.08	13.31[ab]±0.38	12.87[ab]±0.13
Normal cycling	11.61[b]±0.19	12.24[a]±0.98	11.43[c]±0.44	11.70[c]±0.50	11.99[c]±0.59	12.53[b]±0.37	12.36[b]±0.36	12.40[b]±0.36	12.40[b]±0.34
G6PD (U/g Hb)									
Anoesrtus	9.25[a]±0.19	8.52[ab]±0.31	7.51[ac**]±0.25	6.95[a**]±0.23	6.86[a**]±0.22	6.83[a**]±0.25	6.87[ac**]±0.23	6.89[ac**]±0.25	6.87[ac**]±0.22
Anoestrus control	9.24[a]±0.25	9.25[a]±0.24	8.89[b]±0.28	9.47[b]±0.23	9.41[b]±0.21	9.36[b]±0.18	9.37[b]±0.18	9.40[b]±0.15	9.38[b]±0.16
Normal cycling	5.58[b]±0.26	5.29[c]±0.15	5.26[d]±0.34	5.19[c]±0.33	5.20[c]±0.39	5.31[c]±0.45	5.24[d]±0.51	5.24[d]±0.51	5.22[d]±0.53

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.

Glucose-6-phosphate dehydrogenase

The pre-supplementation erythrocytic G6PD activities in anestrus heifers were significantly (p<0.05) higher as compared to those in normal cycling subjects. After supplementation of α-tocopherol, a significant (p<0.01) decline in G6PD activity was observed in anestrus animals with lowest activity at 10th week of supplementation. The period of supplementation and erythrocytic G6PD activities revealed a significant (p<0.05) polynomial regression (R²=0.9867) in anestrus group (Figure 1).

G6PD is a secondary antioxidant enzyme, which catalyzes the first step in glucose metabolism through pentose phosphate pathway (PPP) and generates NADPH from oxidation of glucose-6-phosphate to 6 phosphogluconolactone (Figure 2). Therefore, PPP plays a central role in the metabolic protection against free radicals by generating NADPH (Harvey, 1989), which serve as electron donor in the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) in a reaction catalyzed by glutathione reductase (EC 1.6.4.2; GR). In turn, GSH removes H_2O_2 and ROOH from erythrocytes in a GPX-catalyzed reaction. Vitamin E deficiency caused induction of G6PD activities (Chow et al., 1973; Walsh et al., 1993a) and elevated the tissue concentrations of 4-
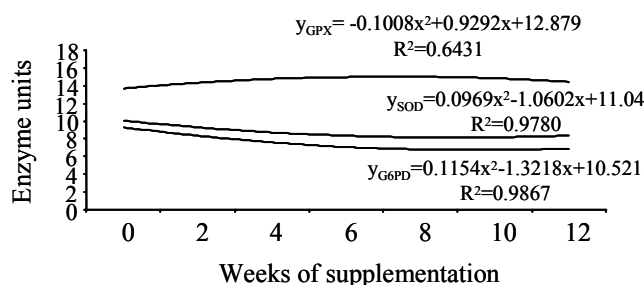

$y_{GPX} = -0.1008x^2 + 0.9292x + 12.879$
$R^2 = 0.6431$
$y_{SOD} = 0.0969x^2 - 1.0602x + 11.04$
$R^2 = 0.9780$
$y_{G6PD} = 0.1154x^2 - 1.3218x + 10.521$
$R^2 = 0.9867$

Figure 1. Regression analysis of erythrocytic SOD (U/mg Hb), GPX (U/mg Hb) and G6PD (U/g Hb) activity in α-tocopherol supplemented anoestrus buffalo heifers.

hydroxynonenal (Walsh et al., 1993b), which is one of the most toxic end product of lipid peroxidation (Esterbauer et al., 1991). Removal and detoxification of 4-hydroxynonenal occurs on conjugation with GSH in a reaction catalyzed by glutathione transferase (EC 2.5.1.18; GSHT; Alin et al., 1985) and the activity of GSHT in vitamin E deficiency was reported to be increased (Lawrence et al., 1978; Mehlert and Diplock, 1985). Therefore, increased activities of G6PD in anestrus buffalo heifers might reflect a physiologic adaptation to maintained the intracellular GSH concentrations essential for removal of H_2O_2, ROOH and 4-

$$2O_2^{\cdot -}$$
$$+$$
$$2H^+ \xrightarrow{\text{SOD}}$$

Figure content:

$2O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 (ROOH)$

$TO^{\cdot}$

$\text{PUSFA} \xrightarrow{\text{Oxidative stress}} ROO^{\cdot} + TOH$

$H_2O_2 (ROOH) \xrightarrow{\text{GPX}} 2H_2O (ROH+H_2O)$

$2GSH$ $\xrightarrow{\text{GR}}$ $2GSSG$

$NADP^+$ $G6PD$

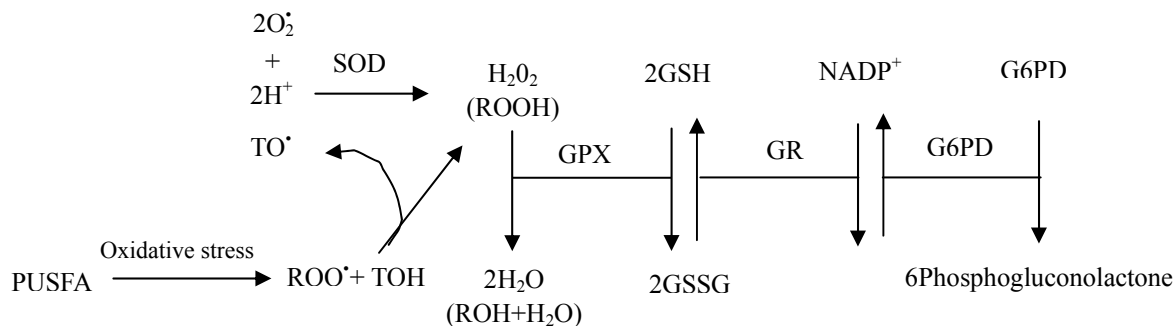$\xrightarrow{\text{G6PD}}$ 6Phosphogluconolactone

Figure 2. Actions of antioxidant enzymes

hydroxynonenal. The decreased plasma vitamin E levels in anestrus (Kahlon, 1999) could be another factor responsible for induction of G6PD activities. Supplementation of α-tocopherol in anestrus animals inhibited erythrocytic SOD activities (Table 1) and lipid peroxidation (Kahlon, 1999), which led to decreased generation of H_2O_2, ROOH and 4-hydroxynoneral. Therefore, a decreased requirement of intracellular GSH during α-tocopherol supplementation might have increased intracellular NADPH, which resulted in inhibition of erythrocytic G6PD.

CONCLUSION

The upregulation of erythrocytic activities of SOD, GPX and G6PD 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 ameliorate antioxidant status as elucidated by decreased activities of erythrocytic SOD and G6PD and increased GPX activity. 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 investigation are, therefore, of immense significance for laying the foundation of physiological norms of antioxidant enzymes in normal cycling and anoestrus buffalo heifers and would be useful for further research in the field of reproductive and nutritional status of buffaloes.

REFERENCES

Alin, P., U. H. Danielson and B. Mannervik. 1985. 4-Hydroxyalk-2-enals are substances for glutathione transferase. FEBS Letters 179:269-270.

Burton, G. W. and M. G. Traber 1990. Vitamin E : antioxidant activity, biokinetics and bioavailability. Annu. Rev. Nutr 10:357-382.

Chow, C. K., K. Reddy and A. L. Tappel 1973. Effect of dietary vitamin E on the activities of the glutathione peroxidase system in rat tissue. J. Nutr. 103:618-624.

Deutsch, J. 1978. Maleimide as an inhibitor in measurement of erythrocyte glucose-6-phosphate dehydrogenase activity. Clin. Chem. 24:885-889.

Esterbauer, H., R. J. Schaur and H. Zollner. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Rad. Biol. Med. 11:81-128.

Ferrell, P. M. and R. J. Robert. 1994. Vitamin E. In: Modern Nutrition in Health and Disease (Ed. M. È. Shils, J. A. Olson and M. Shike). 8th ed, Lea & Febiger, Philadelphia. pp. 326-341.

Hafeman, D. G., R. A. Sunde and W. G. Hoekstra. 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr. 104:580-587.

Harvey, J. W. 1989. Erythrocyte metabolism In: Clinical Biochemistry of Domestic Animals. (Ed. J. J. Kaneko). 4th ed, Academic Press, New York. pp. 185-234.

Jukola, E., J. Hakkarainen, H. Saloniemi and S. Sankari. 1996. Blood selenium, vitamin E, vitamin A and β-carotene concentrations and udder development, fertility treatments and fertility. J. Dairy Sci. 79:838-845.

Kahlon, R. S. 1999. Studies on antioxidant status of normal cycling, delayed pubertal and anesrus buffaloes (*Bubalus bubalis*). Ph.D. dissertation, Punjab Agricultural University, Ludhiana, India.

Kayden, H. J., C. K. Chow and L. K. Bjornson. 1973. Spectrophotometric method for determination of tocopherol in red blood cells. J. Lipid Res. 14:533-540.

Lawrence, R. A., L. K. Parkhill and R. F. Burk. 1978. Hepatic cytosolic non selenium - dependent glutathione peroxidase activity : its nature and the effect of selenium deficiency. J. Nutr. 108:981-987.

Mayes, P. A. 1996. Structure and function of lipid soluble vitamins. In: Harper's Biochemistry (Ed. R. K. Murray, D. K. Granner, P. A. Mayes and V. M. Rodwell). 24th ed, Prentice Hall International limited, London. pp. 614-624.

Mehlert, A. and A. T. Diplock. 1985. The glutathione-S-transferase in selenium and vitamin E deficiency. Biochem. J. 227:823-831.

Nishikimi, M., N. A. Rao and K. Yagi. 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosufate and molecular oxygen. Biochem. Biophys. Res. *Commun.* 46:849-854.

Putnam, M. E. and N. Comben. 1987. Vitamin E. Vet. Rec.

121:541-545.

Rock, C. L., R. A. Jacob and P. E. Bowen. 1996. Update on the biological characteristics of the antioxidant micronutrients : vitamin C, vitamin E and the carotenoids. J. Am Diet. Assoc. 96:693-702.

Rotruck, J. T., A. L. Pope, H. E., Ganther, A. B. Swanson, D. G. Hafeman and W. G. Hoekstra. 1973. Selenium : biochemical role as a component of glutathione peroxidase. Science 179: 588-590.

Walsh, D. M., S. Kennedy, W. J. Blanchflower, E. A. Goodall and D. G. Kennedy. 1993a. Vitamin E and selenium deficiencies increase indices of lipid peroxidation in ruminant calves. Int. J. Vit. Nutr. Res. 63:188-194.

Walsh, D. S., D. G. Kennedy, E. A. Goodall and S. Kennedy 1993b. Antioxidant enzyme activity in muscle of calves depleted of vitamin E or selenium or both. Br. J. Nutr. 70:621-630.

Zoeren-Grobben, D. van, J. H. N. Lindeman, E. Houdkamp, R. Bland, J. Schrijver, H. M. Berger and D. Van-Zoeren-Grobben. 1994. Postnatal changes in plasma chain breaking antioxidants in healthy pre term infants fed formula and/or human milk. Am. J. Clin. Nutr. 60:900-906.

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