**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$_2$O$_2$ 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$_2$•) to H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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.
compared to those in normal cycling subjects. After supplementation of glucose-6-phosphate dehydrogenase (G6PD) activity was significantly (p<0.05) higher as revealed a significant (p<0.05) polynomial regression with lowest activity at 10th week of supplementation. The decline in G6PD activity was observed in anestrus animals. The values having asterisk (** p<0.001; * p<0.05) within a row differ significantly from pre supplementation value. The values having same superscripts within a parameter column don't differ significantly (p<0.05) from each other.

\[ y_{G6PD} = 0.1154x^2 - 1.3218x + 10.521 \]

\[ R^2 = 0.9867 \]

\[ y_{GPX} = 0.0969x^2 - 1.0602x + 11.04 \]

\[ R^2 = 0.9780 \]

\[ y_{SOD} = 0.0969x^2 - 1.0602x + 11.04 \]

\[ R^2 = 0.9780 \]

**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 anoestrous buffalo heifers (Mean±SE)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weeks of supplementation</th>
<th>Weeks of post supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin E (μmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anoestrus</td>
<td>4.06±0.07</td>
<td>4.63±0.05</td>
</tr>
<tr>
<td>Anoestrus control</td>
<td>4.14±0.12</td>
<td>4.29±0.04</td>
</tr>
<tr>
<td>Normal cycling SOD (U/mg Hb)</td>
<td>4.92±0.05</td>
<td>4.94±0.04</td>
</tr>
<tr>
<td>Anoestrus</td>
<td>10.08±0.09</td>
<td>9.40±0.05</td>
</tr>
<tr>
<td>Anoestrus control</td>
<td>10.20±0.06</td>
<td>10.25±0.06</td>
</tr>
<tr>
<td>Normal cycling GPX (U/mg Hb)</td>
<td>6.93±0.04</td>
<td>6.96±0.04</td>
</tr>
<tr>
<td>Anoestrus</td>
<td>14.09±0.54</td>
<td>13.88±0.48</td>
</tr>
<tr>
<td>Anoestrus control</td>
<td>13.47±0.61</td>
<td>14.06±0.14</td>
</tr>
<tr>
<td>Normal cycling G6PD (U/g Hb)</td>
<td>11.61±0.19</td>
<td>12.24±0.98</td>
</tr>
<tr>
<td>Anoestrus</td>
<td>9.25±0.19</td>
<td>8.52±0.31</td>
</tr>
<tr>
<td>Anoestrus control</td>
<td>9.24±0.25</td>
<td>9.25±0.24</td>
</tr>
<tr>
<td>Normal cycling</td>
<td>5.58±0.26</td>
<td>5.29±0.15</td>
</tr>
</tbody>
</table>

**Glucose-6-phosphate dehydrogenase**

The pre-supplementation erythrocytic G6PD activities in anoestrous 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 anoestrous 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 anoestrous 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 phosphoglucose (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 H₂O₂ 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-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 anoestrous buffalo heifers might reflect a physiologic adaptation to maintained the intracellular GSH concentrations essential for removal of H₂O₂, ROOH and 4-
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 H2O2, ROOH and 4-hydroxynonenal. 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**


