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# Assessment of role of antioxidants in erythrocytes of Marwari goat from arid tracts in India to evaluate oxidative stress

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**Abstract**. To assess the role of erythrocytic antioxidants in evaluating oxidative stress, blood samples were collected to harvest erythrocytes from Marwari goat from arid tracts in India. Sampling was carried out in moderate, hot and cold ambiences. Antioxidants determined were vitamins A, E and C and glutathione. The moderate mean values (μmol $gHb^{-1}$) were 0.40±0.01, 0.45±0.02, 2.42±0.05 and 1.44±0.03, respectively. The levels of all the antioxidants decreased significantly ($P<0.05$) during hot and cold ambiences as compared to moderate ambience. The extent of decrease was greater during hot than cold ambience. In hot ambience maximum change was observed in the value of vitamin A (2.35 times). It was followed by vitamin E (1.73 times), vitamin C (1.51 times) and glutathione (1.24 times). In each ambience the effects of gender and age were significant ($P<0.05$) on each antioxidant. It was concluded that extreme ambiences produced oxidative stress in the animals to a greater extent which resulted in depletion of the level of each of the antioxidant in erythrocytes. On the basis of results, supplementation of antioxidants to animals is recommended along with protection from harsh ambience.
**Key Words**: Ambience, vitamin A, cold, hot, Marwari goat.

**Introduction**. Antioxidants are considered as free radical scavengers in the prevention of pathologies in animals in which free radicals are implicated. Reactive free radical species are associated with several forms of tissue damage and disease, and also with the process of ageing. Protection is thought to be available in the form of endogenous compounds that react with and thereby scavenge the reactive oxygen species. To be an effective antioxidant physiologically, a substance must have certain chemical and biological properties. It must be present in adequate amounts in the body and must react with a variety of reactive oxygen species (Rose & Bode 1993).

The inevitability of exposure of animals in arid tracts to extreme hot and cold ambiences makes oxidative stress associated with extreme ambient conditions an appropriate field of investigation to explore adaptive physiological measures of the body and their use in health management and clinical diagnosis (Maan & Kataria 2012).

Ambient stress can reduce antioxidant activity of blood (Harmon et al 1997) resulting in oxidative stress. Glutathione, vitamin E, vitamin C, vitamin A and β-carotene are considered as good antioxidants (Pandey et al 2012).

Oxidative damage to biological systems is the basis of a number of physiological and pathological phenomena. Erythrocytes have often been used as a convenient model for these studies. Erythrocytes are equipped with a variety of biochemical mechanisms operating against cellular damage. One such line of defense is provided by the antioxidant system which helps to detoxify highly reactive species such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals that are being generated during oxidative stress (Gelman et al 1978).

The oxidation process in erythrocytes affects the overall cell structures, hemoglobin and membrane. Several hypotheses have been proposed to explain the mechanism of erythrocytes haemolysis following oxidative stress in vivo and vitro (D' Aguino et al 1983). Hemoglobin appears to be the main site of damage when various

oxidative drugs are used (Goldberg & Stern 1977). Under other oxidative conditions, the membrane appears to be the target of injury leading to hemolysis (Trotta et al 1981).

Certain markers to assess oxidative stress have been established in the form of antioxidants and the levels can increase or decrease due to reactive oxygen species. Heat stress modulates metabolic reactions through free radicals and produces oxidative stress (Kataria et al 2010a). Therefore it is mandatory to determine the levels of antioxidants regulating metabolism in erythrocytes to find out the true picture of metabolic changes. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells along with alteration in the immune status of animals by increasing susceptibility to infectious agents and by reducing the rate of wound healing (Cacioppo et al 2002). The developing oxidative stress can only be detected by laboratory methods. As erythrocytes are likely to be the primary target site for the damage, they may be useful as an early diagnostic tool of oxidative stress. Therefore, in the present study some antioxidants of erythrocytes were chosen to make a comparative evaluation of the extreme ambience induced oxidative stress in Marwari goat from arid tracts in India.

**Material and Method**. To carry out the study, six hundred and thirty apparently healthy Marwari goats of either gender, between 6 months to 4.5 years of age were screened to determine antioxidants in the erythrocytes during moderate, hot and cold ambiences. All the animals were kept in similar conditions of feeding and watering. In each ambience 210 blood samples were collected and the animals were grouped into males (105 individuals) and non pregnant females (105 individuals). Further each group was divided according to age as below 1 year (35 males and 35 females); 1-2 years (35 males and 35 females) and 2-4.5 years (35 males and 35 females). Blood samples were collected in dipotassium EDTA from jugular vein during slaughtering from private slaughter houses (Bikaner, Rajasthan, India). Sampling was carried out in morning hours during moderate (Mean maximum ambient temperature 30.34 ± 0.20 $^{o}$C), hot (Mean maximum ambient temperature 45.58 ± 0.09 $^{o}$C) and cold (Mean minimum ambient temperature 2.05 ± 0.02 $^{o}$C) ambiences.

Erythrocytes were separated by centrifugation (10 minutes at 2700 rpm). Aliquots of erythrocytes were washed twice with isotonic saline solution and then were stored at 20 $^{o}$C until analysis. Erythrocytes were haemolysed with four volumes of ice cold distilled water (Russell et al 1985). For the determination of enzyme activities the haemolysates were treated with equal volumes of ethanol/chloroform (3:5 v/v) mixture. Then centrifuged for 20 minutes at 2700 rpm. This precipitated the haemoglobin and stroma free haemolysate was obtained. This stroma free haemolysate was used to determine the various parameters. On the basis of quantity of haemoglobin present in haemolysate, calculations for various parameters were made. Vitamin A was determined by the method of Varley (1988) with little modification to convert the values to µmol $gHb^{-1}$ on the basis of haemoglobin present in haemolysate. Stroma free haemolysate was treated with alcohol and the retinol was extracted into light petroleum. Then the light petroleum was evaporated and the residue was dissolved in chloroform. Vitamin C was determined by the method as described by Varley (1988) with little modification as follows in calculation.

$$\text{Ascorbic acid (umol L}^{-1}) = \frac{100}{\text{mL titration}} \times 2 \times 0.008 \times 56.78$$

200µl of the dye solution is equivalent to 0.008 mg ascorbic acid. 56.78 is the conversion factor for µmol $L^{-1}$ from mg/dl. Then the values were converted to µmol $gHb^{-1}$.

Vitamin E was determined by the spectrophotometric method of Nair & Magar (1955) with little modification where serum was replaced by stroma free haemolysate and calculations were made according to haemoglobin concentration. Glutathione was determined by the rapid colorimetric micro method of Owens and Belcher (1965) with modifications (Wilson 1968). Sample was treated with phosphate buffer, 5, 5'-dithiobis (2-nitrobenzoic acid), EDTA and glutathione reductase. Addition of $NADPH_2$ to the system

initiates a progressive reduction of 5, 5'-dithiobis (2-nitrobenzoic acid) by glutathione which causes a color increase at 412 mμ. The rate of this change is calculated at 5 minutes interval and is proportionate to amount of glutathione present in sample. Simultaneously standards are processed to form a standard curve to determine the concentrations.

To test the significance of the effects of ambiences, gender and age, the changes in the means were compared by using multiple mean comparison procedures keeping moderate mean as control for each antioxidant (Steel & Torrie 1980).

**Results and Discussion**.

The mean values of erythrocytic vitamins A, E, C and glutathione are shown in Table 1. Vitamin A (Table 1) were significantly ($P<0.05$) lower during hot and cold ambiences as compared to moderate mean value. The decrease was comparatively more in hot than in cold ambience for each antioxidant. The gender and age effects on the mean values of glutathione, vitamin E, vitamin C, vitamin A and β carotene were significant ($P<0.05$) in all ambiences. For each antioxidant, the mean value was significantly ($P<0.05$) higher in male animals than females. Age effect showed a significant ($P<0.05$) increase in the mean values being highest in the 2-4.5 years of animals for each antioxidant.

Table 1

Mean ± SEM values of erythrocytic antioxidants in Marwari goat

| *Effects* | *Antioxidants, μmol gHb-1* | | | |
|---|---|---|---|---|
| | *Vitamin A* | *Vitamin E* | *Vitamin C* | *Glutathione* |
| Moderate (210) | 0.40±0.01 [b] | 0.45±0.02 [b] | 2.42±0.05 [b] | 1.44±0.03 [b] |
| Male (105) | 0.45±0.03 [d] | 0.59±0.02 [d] | 2.82±0.03 [d] | 1.53±0.04 [d] |
| Female (105) | 0.35±0.02 [d] | 0.32±0.02 [d] | 2.02±0.02 [d] | 1.35±0.03 [d] |
| Below 1 year (70) | 0.34±0.01 [f] | 0.27±0.01 [f] | 2.03±0.03 [f] | 1.33±0.04 [f] |
| 1-2 years (70) | 0.40±0.02 [f] | 0.46±0.02 [f] | 2.40±0.03 [f] | 1.45±0.06 [f] |
| 2 -4.5 years (70) | 0.46±0.01 [f] | 0.63±0.01 [f] | 2.83±0.04 [f] | 1.54±0.01 [f] |
| Hot (210) | 0.17 ±0.02 [b] | 0.26±0.02 [b] | 1.60±0.06 [b] | 1.16±0.03 [b] |
| Male (105) | 0.21±0.04 [d] | 0.32±0.03 [d] | 1.92±0.04 [d] | 1.21±0.01 [d] |
| Female (105) | 0.13±0.03 [d] | 0.19±0.02 [d] | 1.28±0.03 [d] | 1.11±0.02 [d] |
| Below 1 year (70) | 0.11±0.02 [f] | 0.15±0.03 [f] | 1.18±0.04 [f] | 1.10±0.03 [f] |
| 1-2 years (70) | 0.18±0.03 [f] | 0.24±0.06 [f] | 1.62±0.02 [f] | 1.16±0.05 [f] |
| 2 -4.5 years (70) | 0.22±0.02 [f] | 0.38±0.02 [f] | 2.00±0.02 [f] | 1.22±0.02 [f] |
| Cold (210) | 0.20±0.01 [b] | 0.34±0.02 [b] | 2.00±0.04 [b] | 1.28±0.02 [b] |
| Male (105) | 0.25±0.01 [d] | 0.40±0.02 [d] | 2.40±0.02 [d] | 1.37±0.01 [d] |
| Female (105) | 0.15±0.02 [d] | 0.28±0.01 [d] | 1.60±0.04 [d] | 1.19±0.03 [d] |
| Below 1 year (70) | 0.14±0.02 [f] | 0.26±0.01 [f] | 1.50±0.03 [f] | 1.17±0.01 [f] |
| 1-2 years (70) | 0.20±0.02 [f] | 0.34±0.02 [f] | 2.00±0.04 [f] | 1.29±0.01 [f] |
| 2 -4.5 years (70) | 0.26±0.01 [f] | 0.43±0.01 [f] | 2.50±0.03 [f] | 1.38±0.04 [f] |

Figures in the parenthesis indicate number of goats, 'b' marks significant ($p\leq0.05$) differences among ambience mean values of a parameter, 'd' marks significant ($p\leq0.05$) differences between male and female mean values of a parameter within an ambience, 'f' marks significant ($p\leq0.05$) differences among mean values of different age groups of a parameter within an ambience.

***Vitamin A***. Antioxidant effect of vitamin A is well documented as it is also used to recycle back the oxidised α-tocopheroxyl radicals to the active reduced forms (Wang et al

1999). A decrease in antioxidant defense leads to oxidative damage of biomolecules (Beckman & Ames 1998). The decreased levels of vitamin A indicated towards the presence of oxidative stress. Dwaraknath & Pareek (1971) also noted effect of gender on vitamin A levels in sheep. The low erythrocytic vitamin A level in the present study in females could be due to higher mobilization for various metabolic purposes (Knig 1961). Taylor et al (1968) reported significant effects of age in both genders on plasma vitamin A.

***Vitamin E***. Vitamin E is considered to be most important lipid-soluble antioxidant, protecting cell membranes from oxidation by reacting with lipid radicals during lipid peroxidation chain reaction (Traber et al 2007). Decreased vitamin E levels have been well correlated with increased oxidative threats (Walwadkar et al 2006). This would remove the free radical intermediates and prevent the oxidation reaction from continuing. In present study serum vitamin E level was lower during hot ambience which showed its depletion in an attempt to reduce the production of reactive oxygen species (Kataria et al 2010b).

Reduced antioxidant activity in heat stressed animals have been studied earlier by researchers (Harmon et al 1997; Calamari et al 1999; Siva Kumar et al 2007), and they attributed it to increased free radical production. Marshall et al (2002) opined that oxidative stress could be provoked by various factors. Stress is one factor, resulting in increased production of free radicals and decreased antioxidant capacity (Lovel 1988; Ishikawa & Kanai 1998). Protective effect of vitamin E was observed by many workers to prevent oxidative stress in erythrocytes and therefore during various types of stresses, lowering of vitamin E levels in erythrocytes were recorded (Kawai et al 2000; Armutcu et al 2005).

Earlier researches have confirmed the depletion of vitamin E in heat stressed animals, as its supplementation increased plasma vitamin E levels (Charmley et al 1992). McDowell (2000) opined that reduction in vitamin E during heat stress might be due to either depletion of endogenous reserves to combat free radicals produced excessively in the body or insufficient endogenous synthesis under stressful conditions. Kataria et al (2010c) also attributed decrease in serum vitamin E to its depletion to combat oxidative stress. It can be concluded that extreme ambiences in present study produced oxidative stress in the goat. Nazifi et al (2009) discussed about the influence of age on free radical generation and consequently, the enzyme antioxidant defense. De & Durad (1991) observed a decrease in vitamin E with the advancement of age. However, in our study vitamin E levels increased with the advancement of age. Age related variation in antioxidant level could be hypothesized on the basis of relationship of free radicals with age (Sastre et al 2000). Vitamin E is also important in the management of stressed animals (Chirase et al 2001).

***Vitamin C***. Vitamin C as an antioxidant protects the body against oxidative stress (Padayatty et al 2003). L-ascorbate is converted to its oxidised form, L-dehydroascorbate, which can then be reduced back to the active L-ascorbate form by enzymes and glutathione. With the help of glutathione, dehydroxyascorbate is converted back to ascorbate. Earlier conducted researches emphasized upon the role of vitamin C in maintaining integrity of erythrocytes by preventing the oxidative stress and suicidal death (Mahmud et al 2010). These results corroborated the earlier findings carried out by Kataria et al (2010b) in dromedaries for serum vitamin C status.

Decreased total antioxidant levels during heat stress (Harmon et al 1997) might be due to increased production of free radicals (Lovel 1988), depletion of endogenous reserves to combat free radicals produced excessively or insufficient endogenous synthesis (McDowell 2000) under stressful conditions. Vitamin C in erythrocytes is suggested to be essential in the detoxification of superoxide radicals and hydrogen peroxide formed during red cell metabolism (Fakhri et al 1991). Depletion of vitamin C confirms the presence of oxidative stress because repletion is reported after supplementation of vitamin C (Hidiroglou 1999; Weiss 2001). Kataria et al (2010c) also recommended the use of antioxidants in the conditions causing oxidative stress. The

observations of present study, showed the depletion of vitamin C to combat free radicals confirming the presence of oxidative stress. Role of vitamin C in preventing oxidative stress of erythrocytes was also studied by earlier workers (Vani et al 2010).

Higher vitamin C level in male animals reflected towards its higher synthesis to combat free radicals (Long et al 1963). Nazifi et al (2009) discussed about the influence of age on free radical generation and consequently, the enzyme antioxidant defense. De & Durad (1991) observed a decrease in vitamin C with the advancement of age. However, in our study vitamin C levels increased with the advancement of age.

***Glutathione***. Cortisol mediated increase in antioxidant levels lead to higher rate of reaction between oxidants and antioxidants, ultimately leading to depletion of antioxidants. Glutathione is an endogenous antioxidant which protects the cells from reactive oxygen species such as free radicals and peroxides (Pompella et al 2003). Glutathione spares ascorbate and improves antioxidant capacity of blood (Gropper et al 2004) and without it dehydroxyascorbate could not convert back to ascorbate. The ratio of reduced glutathione to oxidised glutathione within cells is used as a measure of cellular toxicity (Pastore et al 2003). In present study erythrocytic glutathione level was lower during hot and cold ambiences which indicated its depletion in the process to prevent oxidative stress.

Bernabucci et al (2002) observed that cows exposed to moderate heat stress due to summer temperature showed higher erythrocyte glutathione. These variations could be due to variations in the ambient temperature as in our study the ambient temperature during summer was much higher than their reporting. Depletion of glutathione was correlated to oxidative stress by various earlier workers (Kataria et al 2010a). Effect of environmental temperature on serum glutathione levels was recorded by many earlier researchers in animals, as Dehghan et al (2010) in rams, Kataria et al (2010a) in goats, and Kataria et al (2010b) in camels. This showed that antioxidant defense system was changed to adapt and prevent oxidative stress effects because it protects cells from oxidative damages.

**Conclusions**. The findings clearly reflected the presence of oxidative stress during extreme ambiences. It was concluded that hot ambience affected the animals greatly than cold ambience. It was based upon the fact that degree of depletion of antioxidants in erythrocytes was greater in hot ambience than cold ambience. This indirectly proved that hot ambience produced more free radicals than cold ambience. In hot ambience maximum change was observed in the value of vitamin A (2.35 times). It was followed by vitamin E (1.73 times), vitamin C (1.51 times) and glutathione (1.24 times). On the basis of results, supplementation of antioxidants to animals is recommended along with protection from harsh ambience.

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