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Research Article

OXIDATIVE STRESS IN RED BLOOD CELLS IN HAEMOGLOBINURIC BUFFALOES

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ABSTRACT

Oxidative stress can be measured as thiobarbituric acid reactive species (TBARS). During the present investigation it was estimated in erythrocytes of healthy and haemoglobinuric buffaloes. The erythrocytic mean TBARS in haemoglobinuric buffaloes was significantly higher than healthy buffaloes. The mean TBARS in haemoglobinuric buffaloes without hydrogen peroxide was 1.99 ± 0.23 n moles/ml whereas in the corresponding healthy buffaloes was 1.04 ± 0.30 n moles/ml and in 1.5% hydrogen peroxide the values were 23.49 ± 3.67 n moles/ml and 9.86 ± 1.71 n moles/ml, respectively. High values of total oxidative stress in haemoglobinuric buffaloes suggested free radicals injury to phospholipids membrane of red cells leading to haemolysis

Key words: Oxidative stress, thiobarbituric acid reactive species, buffaloes, haemoglobinuria

In addition to phosphorus deficiency the increase in oxidative stress is also responsible for haemolysis in buffaloes with post-parturient haemoglobinuria (PPH) (Gahlawat, 1998). Oxidative stress is the disturbance in the per oxidant-antioxidant balance in favor of the former. Erythrocyte membrane is rich in polyunsaturated fatty acids and continuously exposed to high oxygen tension, so circulating red cells are one of the most per oxidation susceptible biological tissue (Pryor, 1976). Lipid per oxidation of red cell membrane induced by oxidative attack increases the rigidity of lipid bilayer, which inturn increases the osmotic fragility and eventual haemolysis of red cells in PPH cases.

MATERIALS AND METHODS

The present study was conducted on 50 clinical cases of post-parturient haemoglobinuria along with 30 healthy lactating, non-pregnant buffaloes. Blood samples were collected by jugular venepuncture in sterilized vials containing an anticoagulant. Oxidative stress in erythrocytes was estimated as per the procedures described by Duthie *et al.* (1989) and Bryszewska *et al.* (1995). When oxidative stress increased on erythrocytes, certain alkanes, alkens, alcohols, aldehydes, ketones, etc., were produced in cytosol and membrane. These species give colored reaction with thiobarbituric acid. The intensity of colour was measured spectrophotometrically at 535 nm.

A total volume of 9 ml was made by suspending 1.2 ml red blood cells (RBCs) in normal saline solution. This suspension was equally distributed among six centrifuge tubes and 1.2 ml of H_2O_2 of concentration 0, 0.3, 0.6, 0.9, 1.2 and 1.5% was added in each tube, respectively. The tubes were incubated at 37^0C for 90 minutes in a water bath. The reaction was stopped by adding 0.5 ml 10% trichloro acetic acid (TCA) and tubes were centrifuged at 1500 xg for 10 min. The contents were filtered through Whatman filter paper No.1 and then 0.5 ml filtrate was added in tubes having 0.75 ml of 0.67% thiobarbituric acid. The tubes were placed in boiling water bath for 20 min. After cooling, optical density (OD) was taken at 535 nm in spectrophotometer.

RESULTS AND DISCUSSION

The results regarding mean thiobarbituric acid

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Table
Mean thiobarbituric acid reactive species in healthy and haemoglobinuric buffalo red blood cells

H_2O_2 concentration	Healthy buffaloes	Haemoglobinuric buffaloes
0.0	1.04 ± 0.30	01.99 ± 0.23
0.3	2.55 ± 0.84	03.62 ± 0.56
0.6	3.86 ± 1.22	15.13 ± 0.97
0.9	5.42 ± 1.76	17.68 ± 1.70
1.2	7.58 ± 1.48	19.94 ± 2.49
1.5	8.86 ± 1.71	23.49 ± 3.67

Mean differ significantly between healthy and haemoglobinuric groups (P<0.01)

reactive species (TBARS) are given in the Table. Oxidants produced different patterns of intracellular and membrane damage which may be related to differences in lipid solubility, redox potentials, reactivity with SH groups, binding to haeme and the source or site of oxidant generation. Extracellularly produced oxidants can damage the membrane before reaching the cytosolic protective mechanisms. Oxidants generated intracellularly in coupled reactions with oxyhaemoglobin tend to produce more haemoglobin injury than membrane injury (Harvey, 1997). Rice-Evans (1990) observed that red cell damage by oxidant stress is generally thought to be the end results of either the oxidation of haemoglobin followed by denaturation of met haemoglobin to haemechromes or free radical attack on membrane components such as the poly-unsaturated fatty acid side chains of the membrane lipids, the reduced thiol groups and other susceptible amino acid side chain of membrane proteins.

According to study, it is difficult to pinpoint the source of oxidants. Absence of Heinz bodies in PPH buffaloes ruled out the possibility of excessive oxidative damage of haemoglobin. The above observation is supported by Singh *et al.* (1995) who reported marginally higher met haemoglobin levels in PPH buffaloes as compared with healthy buffaloes. The source of oxidants may be extracellular, which causes more oxidative damage to erythrocyte membrane than cytosolic haemoglobin. Mohamed *et al.* (1988) in Egypt reported continuous feeding of berseem (*Trifolium alexandronum*) in buffaloes suffering from haemoglobinuria. Later on, it was discovered that berseem had 0.45 per cent of saponins on dry matter basis. Similarly Raza-Hasan and Singh (1992) found buffalo erythrocytes very sensitive to lysis in 0.30 to 0.07 per cent saponin solutions.

The source of oxidants is not confirmed but it is conformed that post-parturient haemoglobinuria in buffaloes is due to oxidative injury on red blood cells leading to haemolysis. This view is supported by Mata (1990) and Sridhar and Bhardwaj (1991) who successfully used vitamin C as a therapy in haemoglobinuric buffaloes with 68.5 and 88 per cent recovery rates, respectively. Similarly Chugh (1994) conducted therapeutic trial using vitamin E in the haemoglobinuric buffaloes with 65 per cent recovery rate. Both vitamin C and vitamin E are well known antioxidants.

The present study to know the total oxidative stress on the red blood cell membrane, erythrocytes of PPH and healthy buffaloes were incubated in different concentration of hydrogen peroxide, an oxidant, thus exposing them to oxygen free radical species. A consequence of free radical mediated damage to the poly-unsaturated fatty acid component of cell membrane is the production of lipid hydro peroxides that can be measured as TBARS. It provides an improved method for identifying oxidative stress susceptible animals. In the present investigation, the erythrocyte mean TBARS in PPH buffaloes were significantly (P< 0.01) higher as compared with healthy buffaloes. When red blood cells of healthy and haemoglobinuric buffaloes were incubated in zero per cent hydrogen peroxide, the level of TBARS was 1.04±0.30 and 1.99±0.23 n moles/ml respectively. At 1.5 per cent hydrogen peroxide concentration, the TBARS were measured as 9.86±1.71 and 23.49±3.67 n moles/ml, respectively. This revealed excessive oxidative stress on haemoglobinuric buffalo erythrocytes.

The per oxidation of the membrane lipids is a consequence of many types of cellular injury in which free radical intermediates are produced in excess of local defence mechanisms and has extensively been reviewed (Girotti, 1985, Kappus,

1985, Rice-Evans, 1990). In post-parturient haemoglobinuric buffaloes the levels of serum potassium were estimated by Singh (1976). The serum potassium levels were found to be increased as compared with healthy buffaloes. This clearly indicated that leakage of potassium was under the influence of oxidative stress causing per oxidation of red cell membrane phospholipids.

Rice-Evans (1990) described mechanism of production of TBARS in erythrocytes. It was suggested that lipid per oxidation may be initiated by any primary reactive free radical species that has sufficient reactivity to abstract a hydrogen atom such as hydroxyl radical, ferryl haemoglobin radical, peroxy radical and propagation occurs leading to the formation of lipid monohydroperoxides. Lipid monohydroperoxides are fairly stable and they are catalyzed by transition metals such as low molecular weight iron complexes, met haemoglobin and heme. Cleavage of carbon bonds during lipid per oxidation reactions result in the formation of aldehydic metabolites (malondialdehyde) (Tappel, 1980) or alkenals, such as 4-hydroxy-alkenals (Esterbauer, 1985). Alkens, alkenals or lipid hydroperoxides may be metabolized rapidly, some of these, like lower molecular weight hydro peroxides, aldehydes and 4-hydroxyl-alkenals, can escape from the membrane and can cause disturbances at a distance. So damage of visceral organs (liver, spleen and kidney) as described by Nagpal *et al.* (1968) and Singh (1976) in post-parturient haemoglobinuria might be the result of free radical injury to these organs. Proteins of red blood cell may also be critical target of free radical attack as they are present both inside and outside of these cells in high concentrations. The consequences of such damage may be protein aggregation and cross linking or protein degradation and fragmentation, depending upon the nature of vulnerable proteins and the attacking species (Wolf *et al.*, 1986, Wolf and Dean, 1986) Oxidized proteins become increasingly susceptible to proteolytic attack (Levine *et al.* 1981). So in pathological status radical damage to protein may lead to intracellular accumulation of denatured proteins, of which erythrocytes have no means of disposing them.

Several amino acid crucial for protein and membrane functions are particularly susceptible to radical damage (Butler *et al.* 1988).Lipid alkoxy, peroxy radical or hydroxyl radical may attack on membrane proteins and modify their configuration.Lysine amino acid may be modified by stable products of lipid per oxidation such as Malondialdehyde or 4- hydroxynonenal. Methionine oxidized to Methionine sulfoxide, cystiene to cysteic acid, tryptophan to kynurenine, N-formyle kynurenine, 5-OH tryptophan and phenylalanineto tyrosine in the presence of hydroxyl radical. So it is clear from the above discussion that haemoglobinuric buffalo erythrocytes are continuously exposed to oxidants. This may lead to damage to red cell membrane phospholipids, proteins and amino acid sequence. Very high levels of TBARS in PPH buffalo erythrocytes might be due to free radical injury causing accumulation of intermediary products of membrane phospholipids and proteins disintegration as a consequence haemolysis occurs. To confirm the mechanism of oxidative damage and level of total oxidative stress, further studies in this direction are needed in PPH buffaloes.

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