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## Assessment of oxidative stress and mineral profile in diarrhoeic buffalo calves

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### Abstract

Diarrhoea being the leading cause of death in calves aged less than six months is a major source of economic loss to the dairy industry. The investigation was carried out on clinical cases of diarrhoea in buffalo calves brought to Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Sciences, LUVAS, Hisar as well as from Hisar city and adjoining areas. Thirty clinical cases were selected in the present study based on faecal examination and were divided into four groups. Six healthy buffalo calves' samples were collected and constituted group I. Mean values of major plasma minerals such as calcium, phosphorus and chloride were found to be decreased significantly ( $p < 0.05$ ) indicating decreased absorption due to hypermotility, loss of electrolytes from body and hypersecretory intestinal mucosa. Oxidative stress parameters were also found to be increased significantly ( $p < 0.05$ ) indicating inflammatory reactions due to systemic infections.

**Keywords:** Reduced glutathione, superoxide dismutase, calcium, phosphorus, chloride

### Introduction

Indian economy mainly depends on livestock for its socio-economic as well as rural household development. Diarrhoea, being the leading cause of death in calves is a major source of economic loss to the cattle industry. Cause of diarrhoea may be a variety of pathogens including bacteria, viruses, protozoa and intestinal parasites. Several parasites like *Balantidium coli*, *Strongyle/Strongyloid* spp., *Eimeria* spp. are associated with calf diarrhoea (Bartels *et al.*, 2010 [2]). Diarrhoea causes dehydration, gastroenteritis, body fluid loss and various body fluid changes as well as alterations in various serum metabolites (Edwards and William, 1972) [4]. Loss of body water results in serious metabolic disturbances resulting in loss of water, sodium, potassium, chloride, bicarbonate and also haematological and other biochemical parameters. Though the free radicals are products of normal body cellular metabolism but, if produced in excess that have a damaging tissue effect, cells are protected from such effects by specific endogenous antioxidant systems for ensuring the removal of such free radicals. The relationship between oxidative stress and antioxidant status of the animal is based on steady state concentration of free radicals and the level of antioxidants in the blood which are closely related to the health and the nutritional status of the host (Smith *et al.*, 1998; Gaal *et al.*, 2006; Jens and Ove, 2006) [15, 5, 8]. An antioxidant glutathione is a tripeptide consisting of glutamic acid, cysteine and glycine is widely distributed in living tissues and is maintained there in relatively large amounts as compared to most other soluble low molecular weight components of the tissues. The major part of this tripeptide (99%) is present in the reduced form (GSH) and a very minor part is present in the oxidized form (GSSG) (Srivastava and Beutler, 1969) [18]. It functions in oxidative reduction reactions and in amino acid transport across the cellular membrane, protects and activates thiol dependent enzymes, and is also used as a cofactor for some enzymes. It is considered to be of utmost importance in mitosis, in the functioning of hormones, and as a reactant to detoxification mechanism.

### Materials and Methods Materials

#### Animals

In this study, thirty buffalo calves (1–6 months of age, male and female) suffering from diarrhoea either referred to the Veterinary Clinical Complex, or in the nearby villages of

Hisar district were used for sampling. Samples were collected after 3-5 days of illness. In addition to these, six healthy calves from LUVAS farm were kept as control and constituted Group-I.

### Clinical examination

The most prominent clinical signs among the buffalo with diarrhoea were mild to severe diarrhoea, depression, dullness and depraved appetite. The animals were weak and reluctant

to move. Based on clinical signs, faecal consistency and appearance the type of diarrhoea is presented in Table-1. The animals were screened for presence of parasitic ova by floatation technique. The animals which were found positive for the presence of *Balantidium coli*, *Strongyle* and *Strongyloid* spp., *Eimeria* spp., constituted as Groups II, III and IV respectively as tabulated in Table-2. Animals having profuse diarrhoea but faecal samples were negative for parasitic ova constituted Group-V.

**Table 1:** Various types of diarrhoea and clinical observations associated with it.

Group No.	Appetite	Diarrhoea (Faecal consistency)	Microscopic Faecal examination		
			<i>Balantidium coli</i>	<i>Strongyle/Strongyloid</i> spp	<i>Eimeria</i> spp.
1	Normal	Semi solid and greenish coloured	-ve	-ve	-ve
2	Slight anorectic	Loose and whitish yellow	+ve	-ve	-ve
3	Slight anorectic	Loose with some undigested ingesta	-ve	+ve	-ve
4	Anorectic	Bloody diarrhoea	-ve	-ve	+ve
5	Mild to severe anorexia	Fluidy with greenish discoloration	-ve	-ve	-ve

**Table 2:** Various groups under study and number of animals in each group

Groups	Number of animals in each group	Faecal samples positive for
Group I (Control)	6	None
Group II	3	<i>Balantidium coli</i>
Group III	16	<i>Strongyle/strongyloid</i> spp.
Group IV	3	<i>Eimeria</i> spp.
Group V	8	Non parasitic diarrhoea

### Collection of blood samples

Approximately 5 ml blood was collected from jugular vein aseptically. For separation of plasma and preparation of haemolysate to measure oxidative stress indices, the blood samples collected in centrifuge tube containing heparin were centrifuged at 3000 rpm for 10 minutes; the plasma was separated in aliquots and buffy coat layer was removed to harvest the red blood cells. After that, red blood cells were washed thrice in an ice-cold normal saline solution (NSS). A part of RBC pellet was diluted with ice-cold distilled water in 1:1 dilution for the preparation of stock haemolysate. The plasma and haemolysate were stored in liquid nitrogen in aliquots till further analysis for estimation of biochemical parameters.

### Methods

#### The oxidative stress indices analysed are as follows Superoxide dismutase (SOD)

The activity of SOD in RBC haemolysate was measured by the method of Madesh and Balsubramaniam (1998) [10]. The assay was based on the generation of superoxide by pyrogallol autoxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye MTT [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] to its formazan, measured at 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO) which also helps to solubilize the formazan formed and the colour developed was stable for hours.

For estimation of SOD activity 0.65 ml of PBS and 30 µl of MTT was added to each of the tubes marked as sample,

control and blank. 10 µl of haemolysate was added to the sample tube. After that 75µl of pyrogallol was added to all the tubes and were incubated for 5 minutes at room temperature. Then 0.75 ml of DMSO was added to all the tubes and 10 µl haemolysate was added to the control tube. The absorbance was read at 570 nm. The activity of the SOD was calculated as follows

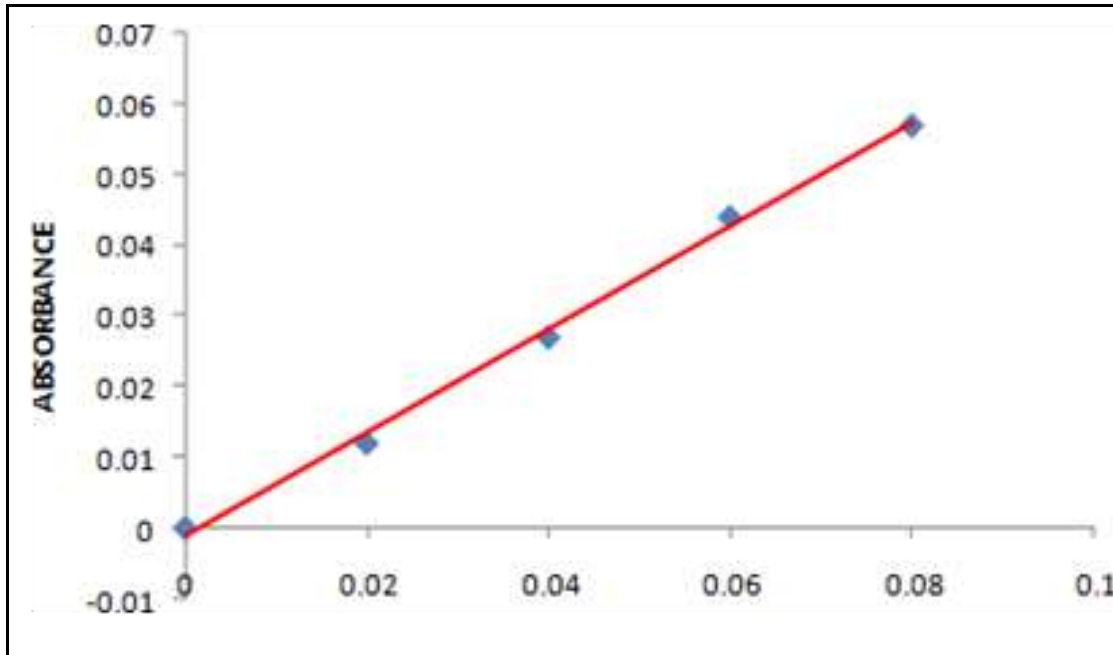
$$\text{SOD (units/mg of protein)} = \frac{\text{mg of protein in 0.01 ml haemolysate}}{\text{y\%}} \times 50 \times \text{DF}$$

$$y\% = \frac{\text{OD of Test}}{\text{OD of Control}} \times 100 \quad [y\% = \% \text{ inhibition of MTT reduction by SOD protein}]$$

[DF = dilution factor]

#### Reduced glutathione

Reduced glutathione in haemolysate was determined as per the procedure described by Beutler (1971) [3]. Reduced glutathione reduces 5-5 -dithiobis (2-nitrobenzoic acid) (DTNB) a disulfide compound, forming highly coloured yellow anions. The absorbance of this yellow compound is measured at 412 nm. For the determination of GSH, 0.5 ml of RBC haemolysate was pipetted into 4 ml of 0.08 N sulfuric acid in a test tube and mixed carefully. After 10 minutes of standing at room temperature, 0.5 ml of tungstate solution (0.3 M Na<sub>2</sub>WO<sub>4</sub> and 0.1 M EDTA) was added to clear brown haemolysate. The mixture was shaken vigorously for 5 minutes. Then it was allowed to stand for few minutes. The suspension was then centrifuged for 10 minutes at 2000g at room temperature. 2 ml of clear extract was pipetted into 2.5 ml of tris buffer, pH 8.0 [1 M tris (hydroxymethyl) aminomethane, pH adjusted to 8.0 with HCl] and 0.2 ml of the DTNB reagent [0.14 M Na<sub>2</sub>HPO<sub>4</sub>, 0.00013 M NaH<sub>2</sub>PO<sub>4</sub>, and 40mg/100ml 5-5 -dithiobis (2-nitrobenzoic acid)] was added. After 30-60 seconds, the optical density was measured at 412 nm against water. A reagent blank was also prepared using 2 ml distilled water. A reagent blank was also prepared using 2 ml distilled water as substitute for extract. Substituting for the extract 2 ml of standard solution containing 0.02 to 0.1 mM GSH and 0.1 M EDTA prepared a standard curve.



**Fig 1:** Reduced Glutathione (mM)

**Standard curve of reduced glutathione Estimation of plasma minerals Calcium**

Calcium concentration was measured by O-cresolphthalein complexone (OCPC) method by using commercial kit. OCPC reacts with calcium in alkaline medium to form a purple coloured complex. The intensity of the purple colour formed is proportional to the calcium concentration and absorbance is measured photometrically at 575 nm.

**Phosphorus**

Its concentration was measured by Ammonium Molybdate method. In this method inorganic phosphorus combines with ammonium molybdate in the presence of strong acids to form phosphomolybdate. The formation of phosphomolybdate is measured at 340 nm and is directly proportional to the concentration of inorganic phosphorus present.

**Chloride**

Chloride was measured by kit based Ferric Thiocyanate method. In this procedure, when chloride is mixed with a solution of undissociated mercuric thiocyanate, the chlorine preferentially combines with mercury forming

mercuric chloride. The thiocyanate released, combines with ferric ions present in the solution to form strongly coloured ferric thiocyanate with absorption at 480 nm.

**Statistical analysis**

The mean of different parameters was subjected to statistical analysis as outlined by Snedecor and Cochran (1994)<sup>[16]</sup>. Difference of significance in variables among five groups was compared with the help of one-way analysis of variance (ANOVA) in SPSS computer software.

**Results**

**Oxidative stress indices Superoxide dismutase**

Due to diarrhoea mean value of SOD was decreased significantly to 5.92±0.34, 5.34±0.06, 4.84±0.06, 4.67±0.05 U/mg Hb in all diarrheal groups II to IV whereas its level was 6.49±0.23 U/mg Hb in healthy control calves.

**Reduced glutathione**

The mean level of reduced glutathione was 18.59±1.27 (mg/dl) in healthy control group whereas it was significantly decreased in all diarrheic groups. Its values in different groups were 16.13±0.08, 16.39±0.25, 13.23±1.68, 15.97±0.46 (mg/dl) in groups I to V respectively.

**Table 3:** Changes in levels of oxidative stress indices (Mean±SE) in buffalo calves.

Parameters	Group I (n=6)	Diarrhoeic animals (n=30)			
		Group II (n=3)	Group III (n=16)	Group IV (n=3)	Group V (n=8)
SOD(U/mg Hb)	6.49±0.23 <sup>d</sup>	5.92±0.34 <sup>c</sup>	5.34±0.06 <sup>b</sup>	4.84±0.06 <sup>a</sup>	4.67±0.05 <sup>a</sup>
Reduced Glutathione(mg/dl)	18.59±1.27 <sup>c</sup>	16.13±0.08 <sup>b</sup>	16.39±0.25 <sup>bc</sup>	13.23±1.68 <sup>a</sup>	15.97±0.46 <sup>b</sup>

Values with common superscripts do not differ significantly between groups at  $p < 0.05$ . Gp - I, Healthy control; Gp -II, *Balantidium coli* positive; Gp- III, *Strongyle/Strongyloid* spp. positive; Gp- IV, *Eimeria* spp. positive; Gp -V, Non parasitic diarrheal group.

**Plasma minerals**

Due to diarrhea, the mean levels of calcium decreased significantly ( $p < 0.05$ ) in all infected groups as compared to 9.72±1.15 (mg/dl) in healthy control group and it was 8.63±0.72, 8.53±0.67, 8.27±0.20, 8.51±0.83 (mg/dl) in groups II to V.

The mean level of phosphorus was 4.75±0.29 in healthy control group and its level was found to be significantly

decreased ( $p < 0.05$ ) in all diarrhoeic groups where its levels were 4.15±0.06, 4.18±0.59, 3.72±0.22, 3.83±0.63 mg/dl in groups II to V respectively.

Chloride level in healthy control group was 100.58±2.15 mEq/l in healthy control group whereas due to diarrhea it decreased significantly ( $p < 0.05$ ) in all group

**Table 4:** Changes in levels of minerals (Mean±SE) due to diarrhoea in buffalo calves.

Parameters	Group I (n=6)	Diarrhoeic animals (n=30)			
		Group II (n=3)	Group III (n=16)	Group IV (n=3)	Group V (n=8)
Ca(mg/dl)	9.72±1.15 <sup>a</sup>	8.63±0.72 <sup>b</sup>	8.53±0.67 <sup>b</sup>	8.27±0.20 <sup>b</sup>	8.51±0.83 <sup>b</sup>
P(mg/dl)	4.75±0.29 <sup>b</sup>	4.15±0.06 <sup>ab</sup>	4.18±0.59 <sup>ab</sup>	3.72±0.22 <sup>a</sup>	3.83±0.63 <sup>a</sup>
Cl(meq/l)	100.58±2.15 <sup>a</sup>	92.27±3.65 <sup>ab</sup>	94.36±3.73 <sup>b</sup>	89.68±1.17 <sup>c</sup>	91.16±2.18 <sup>ab</sup>

Values with common superscripts do not differ significantly between groups at  $p < 0.05$ .

Gp - I, Healthy control; Gp -II, *Balantidium coli* positive; Gp- III, *Strongyle/Strongyloid* spp. positive; Gp- IV, *Eimeria* spp. positive; Gp -V, Non parasitic diarrheal group.

## Discussion

The level of oxidative stress indices indicates the antioxidant status of the animal. In this study the level of SOD and reduced glutathione were decreased significantly in all diarrhoeic groups as compared to control group. This might be because of increased generation of free radicals under different types of diarrhoea as the free radicals can overwhelm protective systems within the body, producing cellular injury or destruction (Valiko *et al.*, 2007) [19]. The oxidants so generated can vary in their overall reactivities and some are fairly selective for the certain biomolecules (e.g. tyrosine, glutathione, linoleic acid and ascorbate) (Proyer *et al.*, 2006) [13]. As reduced glutathione occupies the central importance in protection against injury, may act non enzymatically as a free radical acceptor to counteract oxidant damage (Prins and Loss, 1969) [12]. GSH can also bind free hemin (containing iron as  $Fe^{+3}$ ) that may be released during Hb oxidative denaturation and helping in reducing the potential of hemin for membrane injury. GSH levels in the present study can also have an effect on various reductive enzyme reactions as GSH functions in donating the electrons to glutathione peroxidase ( $GP_x$ ), Glutathione-S-transferase, and glutaredoxin. In the present study *Eimeria* spp. Infection had the larger effect on reducing the concentration of GSH. Superoxide dismutase, which is a copper and zinc containing enzyme promotes the dismutation of two  $O_2^-$  molecules to  $H_2O_2$  and  $O_2$  thereby preventing the build up superoxide by acting as an oxidant by itself (Pryor *et al.*, 2006) [13]. In the present study, infections causing diarrhoea had significant effect on reduction in the levels of SOD in RBC haemolysates. Similarly, Ghanem *et al.*, 2012 [6] reported the reduced concentrations of SOD in cases of diarrhoea caused by *E. coli*. in buffalo calves. Since SOD degrades the superoxide into oxygen and  $H_2O_2$  which are less toxic substances, its low levels leads to accumulation of oxidant substances and free radicals that cause cellular damage to the intestinal mucosa. The result signifies the important role of antioxidants as supportive agents during calf diarrhoea.

The level of calcium decreased significantly in all diarrheal groups as compared to healthy control group. The possible reason for this may be due to the loss of calcium through faeces because during increased gastro-intestinal motility, absorption cannot occur properly. Similar findings have been reported by Zilaitis *et al.*, 2015 [20]. Diarrhoea caused decrease in levels of phosphorus significantly in all parasitic groups as compared to healthy control group. This may be due to loss of major portion of electrolytes from the body. A reduced value of serum chloride was observed in diarrhoeic calves as compared with the normal healthy calves which is in conformity with data as reported by many workers (Hartmann *et al.*, 1983; Maach *et al.*, 1992; Aly *et al.*, 1996) [7, 9, 1]. However, the present findings are contradictory to the reports of some previous studies in which hyperchloraemia have been observed in diarrhoeic calves (Sridhar *et al.*, 1988) [17]. Hyperchloraemia was reported to occur as a result

of prolonged increased loss of  $Cl^-$  ions in the intestinal tract during diarrhoea (McSherry and Grinyer, 1954; Radiostits *et al.*, 2007) [11, 14], and failure of gastric  $H^+$  and  $Cl^-$  ions to be reabsorbed by the villi of small intestine (Radostits *et al.*, 2007) [14].

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