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Evaluation of oxidative stress in helminthiasis infested horses

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Abstract
The aim of this study was to determine the concentrations of oxidative stress parameters in horses, on the basis of faecal examination, eight horses were diagnosed positive for helminthiasis. The blood samples were collected from these cases for estimation of oxidant and anti-oxidant levels. Malondialdehyde (MDA) was the Oxidant which was estimated and was found increased. Reduced glutathione (GSH), Catalase (CAT), Superoxide dismutase (SOD), Vitamin C and Vitamin E were the Anti-oxidants which were estimated and were found decreased.

Keywords: Horses, faecal examination, helminthiasis, oxidant, anti-oxidants

Introduction
Parasitic diseases can cause oxidative stress since hosts produce reactive oxygen species (ROS), primarily to attack invading pathogens (Dimitrijevic *et al*. 2012, Esmaeilnejad *et al*. 2014) [10, 13]. Despite their protective role, these reactive molecules cannot distinguish between host cells and infectious agents (Sorci and Faivre, 2009) [24]. Under these circumstances, the host activates protective mechanisms including antioxidative defences. If the host is over-whelmed by ROS, oxidative stress may occur (Rahal *et al*. 2014) [21]. Overproduction of ROS can induce oxidative modifications in cellular macromolecules such as lipids, proteins and DNA (Trachootham *et al*. 2008) [25]. Lipid peroxidation of cell membranes by ROS leads to progressive damage and loss of selective permeability. Oxidative damage of proteins can result in loss of biochemical functions, while oxidative DNA damage can cause DNA mutations, replication errors, genomic instability and cell death (Klaunig *et al*. 2010) [18].
Parasites can have detrimental effects on host fitness and infection typically results in the stimulation of the immune system (Alonso-Alvarez *et al*. 2004, Costantini and Moller 2009) [2, 7], while defending against infection, the immune system generates toxic oxidants; if these are not sufficiently counteracted by the antioxidant system, a state of oxidative stress can occur (Van-de *et al*. 2010) [26].
Oxidative stress has been defined as a disturbance of the equilibrium between antioxidants and oxidants in favour of oxidants (Sies, 1985) [22]. It has been implicated as harmful because oxygen free radicals attack biological molecules such as lipids, proteins, and DNA. However, oxidative stress also has a useful role in physiologic adaptation and in the regulation of intracellular signal transduction (Yoshikawa and Naito, 2002) [29]. It is proposed that electrons leaking from the electron transport chain (ETC) produce reactive oxygen species (ROS) so these molecules can damage ETC components and mitochondrial DNA, leading to further increase in intracellular ROS levels and a decline in mitochondrial function (Wallace, 2005).

Materials and Methods
The present study was conducted on eight horses between 4-6 years of age, in and around Jaipur area. On the basis of faecal examination, eight horses were diagnosed positive for helminthiasis (Strongyloides, Parascaris, Anoplocephala). The blood samples were collected from these positive cases from the jugular vein directly into vacuum tubes, and sodium heparin was used as an anticoagulant. These samples were then subjected to laboratory analyses to determine the levels of the following MDA, CAT, SOD. Besides these Vit. C and Vit. E. were also evaluated. EDTA was used as an anticoagulant for GSH.
Samples were centrifuged at 1,900 x g for 15 min at 4 °C to separate the plasma from the red blood cells. The plasma was aliquoted into 1.5 mL Eppendorf tubes. Red blood cells were resuspended in phosphate-buffered saline (PBS) and centrifuged.

This washing procedure was repeated three times, and the red blood cells were stored in Eppendorf microtubes. For the catalase measurements the red blood cells were stored in an EDTA solution and mercaptoethanol. The haemoglobin concentration was determined using the Drabkin's method (Beutler, 1975), where the presence of this reagent forms a compound, cyanmethaemoglobin, which absorbs at an absorbance of 540nm (Celm E225, Brazil).

Oxidant Analysis

Determination of lipid peroxidation

Malondialdehyde (MDA) a secondary product of lipid peroxidation, the reaction of lipid peroxides with Thiobarbituric acid (TBA) yields red pigment which can be measured on Colorimeter or Spectrophotometer at 532 nm. The plasma MDA levels were determined by adding 2mL of 10% trichloroacetic acid (TCA) to 1mL plasma, and the samples were then centrifuged for 15 min at 15°C. Then 750µL of 1% thiobarbituric acid (TBA) was added to the 750µL of supernatant, and the samples were heated in a boiling water bath for 10min. The tubes were cooled in ice water and the absorbance reading was collected using a spectrophotometer (Celm E225, Brazil) at 532nm (Esterbauer and Cheeseman, 1990) [14].

Anti-Oxidant Analysis

Reduced glutathione in blood

Reduced glutathione (GSH) was determined by adding 1.8 mL of purified water to 200µL of whole blood and 3mL of precipitant. After a 5min rest the solution was centrifuged at 1,800 x g for 5min. Then 800µL of phosphate solution and 100µL of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) were added to the 200µL of supernatant, and the samples were immediately read using a spectrophotometer at 412nm (Celm E225, Brazil) (Beutler *et al*. 1963) [5].

Catalase

Catalase activity was measured according to the modified Aebi method (Aebi, 1984) [1], by adding 20µL of haemolysate to 980mL of 50mmol/L phosphate buffer containing 10mmol/L hydrogen peroxide. The absorbance was read using a spectrophotometer at 240nm.

Estimation of Superoxide Dismutase

Estimation of plasma Superoxide Dismutase (SOD) was done by the method of Kakkar *et al.* 1984. 1.35 ml of double distilled water, 50 µl of plasma, 1.2 ml of sodium pyrophosphate buffer (pH 8.3), 0.1 ml of phenazine methosulphate (PMS) and 0.3 ml of nitroblue tetrazolium (NBT) were mixed. 0.2 ml of NADH solution was added to it to initiate the reaction. After incubation at 39°C for 90 s the reaction was terminated by adding 1 ml of glacial acetic acid. 4 ml of *n* -butanol was added and the mixture was centrifuged at 4000 rpm for 10 min and the absorbance of the upper butanol layer recorded at 560 nm. For the comparison, corresponding blank was prepared in the same way except addition of the plasma. One unit of SOD was defined as that amount of enzyme that inhibits the rate of reactions by 50% under specified conditions.

Estimation of Vitamin C

Ascorbic Acid level was estimated by the method of Omaye *et al.* 1979. Ascorbic acid is oxidized by copper to form dehydro-ascorbic acid and diketoglutaric acid. These products when treated with 2,4 Dinitrophenyl hydrazine (DNPH) form the derivative, bis-2-4-dinitrophenylhydrazine which undergoes rearrangement to form a product with 1.0 ml of the plasma was mixed thoroughly with 1.0 ml of ice cold 10% TCA and centrifuged for 20 minutes at 3500xg. To 0.5ml of the supernatant, 0.1ml of DTC reagent was added and mixed well. The tubes were incubated at 37ºC for 3 hrs. 0.75 ml of ice cold 65% sulphuric acid was added and the tubes were allowed to stand at room temperature for an additional 30 minutes. A set of standards containing 10-50 mg of ascorbic acid was processed similarly along with a blank containing 0.5ml of 10%TCA. The colour developed was read at 520nm.

Estimation of Vitamin E

Plasma α-tocopherol was estimated by the method of Baker and Frank, 1968. The method involves the reduction of Ferric ions to Ferrous ions by alpha tocopherol and the formation of a red coloured complex with 2,2" dipyridyl. Absorbance of the chromophore was measured at 520nm. 0.5ml of plasma, 2 ml of petroleum ether and1.6ml of ethanol were added, mixed and centrifuged. To the supernatant 0.2ml of 2,2" dipyridyl solution and 0.2ml of Ferric chloride solution were added, mixed well and kept in the dark for five minutes. An intense red colour was developed. 4 ml of water was added to all the tubes and mixed well. Standard alpha tocopherol in the range of 10 -100 µgms were taken and treated similarly along with a blank containing only the reagent. The colour in the aqueous layer was read at 520nm.

Table 1: Levels of Oxidant and Anti-oxidants in helminthiasis infested horses.

Parameters	MEAN ± SE
Malondialdehyde (nmol/ml)	20.27 ± 1.92
Reduced glutathione (mg/dl)	9.74 ± 0.80
Catalase (KIU/ml)	3.11 ± 0.20
Superoxide dismutase (ng/ml)	0.99 ± 0.15
Vitamin C (mg/dl)	0.56 ± 0.18
Vitamin E (mg/dl)	1.47 ± 0.12

Results and Discussion

Parasitic infections cause an activation of inflammatory cells which play an important role in the host defense. Activation of inflammatory cells induces and activates various oxidant-generating enzymes. In the present study higher level of Malondialdehyde and lower levels of GSH, CAT, SOD, Vit. E and Vit. C (Table 1) were found in blood of helminthiasis infested animals. The increase of MDA might be due to increased lipid peroxidation in the cells (El-Badry 2006, Deger *et al*. 2008 and El-Moghazy 2011, Aziz *et al*. 2018) [11, 9, 12, 3]. In the present study lowered value of reduced glutathione and Catalase in the helminthiasis infested horses might be due to compensatory mechanism against reactive oxygen species (El- Moghazy 2011) [12]. Antioxidant enzymes such as SOD, CAT are important for cellular protection due to their ability to detoxify free radicals, such as ROS (Cosper and Wakefield, 1975) [8]. Cellular non-enzymatic antioxidants are also known as free radical scavengers that protect a cell against toxic free radicals. Vitamin C and E is the chief constituent of the aqueous and lipid soluble environment. Therefore, decreased vitamin C and E may reflect a depletion of non-enzymatic antioxidant reserves. On the other hand, they play prominent role in the antioxidant defense system, and in the reactions of catalysis, regulation, electron transportation and in preserving the correct structure of

proteins. Vitamin E is the most efficient antioxidant in preventing lipid oxidation in lipoproteins (Kagan *et al*. 1990) [16] and is the most commonly supplemented antioxidant in horses (Williams, 2013) [28]. The NRC (2007) [19] recommends 100 IU/d of vitamin E for every 100 kg of BW for the adult horse as maintenance, and this increases to 200 IU/d for every 100 kg of BW for horses in heavy to very heavy work and those in lactation. Membrane concentration of α-tocopherol is approximately 1 α-tocopherol molecule to 1,000 lipid molecules. The phytyl tail of the tocopherol molecule allows the positioning of the molecule within the membrane bilayer so that the active chroman ring lies close to the surface of the membrane (Esterbauer *et al*. 1991) [15]. increase in ROS. It was found in various species that vitamin C potentiates the effects of vitamin E by reducing the tocopheroxyl radical and restoring its activity (Chan, 1993) [6]. Under maintenance conditions, horses have the ability to synthesize sufficient ascorbate, which is why no requirement has been determined (NRC, 2007) [19], but demand increases as stress on the body is increased. Vitamin C is generally measured as ascorbate by HPLC; its antioxidant function is mainly to reduce α-tocopherol and peroxyl radicals. The normal range of ascorbic acid for healthy horses not supplemented with vitamin C is 6 to 10 µg/mL. Normal supplementation of vitamin C to horses is 5 g/d, which increases plasma ascorbic acid concentration by 2 to 3 µg/mL (Snow *et al*. 1987) [23].

Conclusion

The present study revealed that helminthes significantly increases lipid peroxidation as shown by increased MDA levels and decreased levels of CAT, SOD, GSH, Vit. C and Vit. E in infected horses. These markers of oxidative stress could be involved in the increased number of helminthes in the infested horses.

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