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Investigation of the Effects of Carrying Heavy Load on Prooxidation/ Antioxidant Status and Vitamin D_3 in Healthy Horses

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Abstract: The aim of the study was to investigate the effect of carrying heavy load for a long time on lipid peroxidation (MDA: malondialdehyde), NO_2 (nitrite), NO_3 (nitrate), antioxidants (GSH: reduced glutathione, retinol, α-tocopherol) and vitamin D_3 in healthy horses. Blood samples from seventeen native 3-5 years age and 450-500 kg live weight Anatolian horses carried a load which comprised at least 30% of their body weight and for 4 h on mountainous terrain (hard working) were evaluated. Blood samples were collected in the morning before the animals started to carrying load and immediately after they finished carrying (working). It is observed that the level of MDA, NO_2 and NO_3 increased significantly ($p<0.05$) after working. While GSH concentration, increased after working; levels of retinol, α-tocopherol and vitamin D_3 levels decreased significantly ($p<0.05$). On the other hand, the vitamin D_3 levels were affected by hard working as other lipid soluble vitamins. There were a correlation between the physiological response to hard-working and some oxidant markers in healthy-hard working horses. These observations provide evidence that hard-working increases oxygen consumption and cause a disturbance of intracellular pro-oxidant-antioxidant homeostasis.

Key words: Antioxidants, lipid peroxidation, vitamin D_3, packing horse

INTRODUCTION

The horses are used to haul things in rural region. These animals are loaded heavily to carry long distances. This could be considered as heavy exercise. Exercise is known to exert numerous physiological changes in vital organ system of the body. Energy consumption and metabolic activity increases considerably due to the increase of muscle contractions in physical exercise and works. Oxygen consumption increases 10-15 times in exercises with the mitochondrial formation of oxygen and hydrogen peroxide. Free radical formation can also increase under conditions of altered metabolism, ischemia-reperfusion and exercise. The relationship between physical activity and oxidative tissue damage has been investigated in numerous studies in men and animals. Increased metabolism can lead to an increase in free radicals produced by the electron transport system, which in turn leads to a response by cellular antioxidant systems. Evidence of oxidative stress in horses has been described in reports dealing with intense and endurance exercise. Malondialdehyde is the organic compound that occurs naturally and is a marker for oxidative stress (Ji, 1999; Sen and Packer, 2000; Marlin *et al*., 2002; Williams *et al*., 2005).

An important role of nitric oxide (NO) in stress is adaptive responses of organisms and thereby expands the existing notions on the biological role of this unique molecule (Malyshev and Manukhina,

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1998). Recent publications demonstrate that NO possesses the qualities of a biological messenger and modulates neurotransmission. NO is a biologic mediator in biochemical reactions and physiologically, it is synthesized from L-arginine by NO synthase employing cofactor NADPH. In the body, the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) within a very short period of time. This short duration in the conversion of NO to nitrite (NO_2^-) and nitrate (NO_3^-) makes it difficult to accurately measure the concentration of NO. Therefore, by determining the amounts of NO_2 and NO_3 the levels of NO can be assessed. Blood nitrate and nitrite are produced from nitrogen monoxide and its fluctuation may reflect on the fluctuation of nitric oxides. It has been widely reported that the concentration of NO synthesized physiologically increase in some pathologic circumstances and it acts as a free radical (Moncada *et al*., 1991; Bredt and Snyder, 1994; Torreilles and Guyerin, 1995). The stress reaction is characterized by increased exhaled NO is released from the airways of the horse (Mills *et al*., 1996).

Under normal conditions, excessive formation of free radicals and concomitant damage at cellular and tissue concentrations is controlled by enzymatic or non-enzymatic mechanisms, including vitamin E and glutathione (Sies, 1991).

Vitamin D is a steroid prohormone yielding the active hormone derivative calcitriol, which regulates calcium and phosphate metabolism. Vitamin D_3 is synthesized in the skin during summer under the influence of ultraviolet light of the sun, or it is obtained from food, especially fatty fish. Muscle cells contain vitamin D receptor and several studies have demonstrated that serum 25(OH)D is related to physical performance (Lips, 2006).

The aim of the present study is to investigate the effect of exhaustive hard-working on lipid peroxidation (MDA, NO_2, NO_3), antioxidants (GSH, retinol, α-tocopherol) and vitamin D_3 in healthy horses.

MATERIALS AND METHODS

Animals and Data Collection

Seventeen native clinically healthy Anatolian horses (3-5 years age, 450-500 kg live weight and to be used for carrying load at rural region of country) were utilized. They were housed in owners' barn without any additional treatment. They were fed routinely *ad libitum* with both forage and concentrated (barley+dry grass+lentil straw) feed. No supplementary feed was added to the diet. This study was performed in May, 2008 in the region of Van.

Two blood samples were taken from the horses. The first one was taken while animals were at completely rest prior to carrying heavy load. Horses were loaded with load which comprised at least 30% of their body weight and made carried this load for 4 h on mountainous terrain. From here, the term working will be used to describe the activity of carrying heavy load for a long time and at a long distance. The second blood was taken immediately after arriving at the destination.

Blood Sampling and Biochemical Analysis

Blood samples were collected from *V. jugularis* into heparinised tubes. Biochemical parameters were analyzed in the same day. The concentration of whole blood for reduced glutathione (GSH) (Beutler *et al*., 1963), malondialdehyde (MDA) (Jain *et al*., 1989) and the NO oxidation products (Sthar, 1977) were determined by spectrophotometric methods. Vitamin E (α-tocopherol), vitamin A (retinol), vitamin D_3 concentrations were analyzed by HPLC (Zaspel and Csallany, 1983; Reynolds and Judd, 1984; Miller and Yang, 1985).

Statistical Analysis

The results were expressed as Means±SD. Duncan's test was used for statistical analysis, setting $p<0.05$ to establish statistically significant differences.

RESULTS AND DISCUSSION

It is observed that the level of MDA which is the lipid peroxidation marker and the nitrate and nitrite which are the nitric oxide oxidation products increased significantly ($p<0.05$) after working. While GSH concentration, which is the antioxidant system is increasing after working; levels of retinol, α-tocopherol and vitamin D_3 levels were decreased significantly ($p<0.05$) (Table 1).

Although there are plenty of studies in different animal species and human regarding to the effects of exhaustive exercises on antioxidant and lipid peroxidation to the best of our knowledge there is no study on these parameters in horses carrying out hard jobs.

Plenty of evidence indicating that exhaustive exercise generates free radicals has been reported recently. Oxidative stress during endurance exercise as represented by an association between increased lipid peroxidation. Increase of lipid hydroperoxide (LPO) plasma levels in sport horses after competition is an indicator of competition-induced lipid peroxidation. Exercise induced an increase in LPO concentration of horses (Ji, 1999; Williams *et al*., 2005).

There is growing evidence to support the theory that increased oxygen consumption during exercise increases free radicals causing oxidative stress (Sen and Packer, 2000; Balogh *et al*., 2001). It's been reported that MDA and TBARS level increased significantly after exercise in horse (Chiaradia *et al*., 1998; Avellini *et al*., 1999). In this study, the MDA levels increased statistically and this result is compatible with the literature results.

Nitric oxide (NO) plays an important role in stress and adaptive responses of the organism. NO blockade increases metabolic rate, reducing mechanical efficiency during exercise and NO production increases after acute exercise. It is known that adaptation to exercise enhances the organism resistance to acute hypoxia. NO may play a role in the development of the antihypoxic effect of adaptation to exercise (Trolin *et al*., 1994; Malyshev and Manukhina, 1998; Lacerda *et al*., 2006). In this study, it was observed that the concentrations of nitric oxide oxidation products (NO_2, NO_3) increased significantly. As it is in MDA, these parameters increase show that lipid peroxidation and cell damage occur due to the oxidative stress at blood serum in hard-working horses.

Thiols serve as regulators of molecular responses to oxidants. Blood GSH oxidation appears to be a consistent response to physical exercise in humans (Meister, 1995). Experimental studies show that glutathione metabolism in several tissues sensitively responds to an exhaustive exercise. Study of glutathione-deficient animals clearly indicates the central importance of having adequate tissue glutathione to protect against exercise-induced oxidative stress (Sen and Packer, 2000).

While, the GSH concentration in blood and plasma decrease after acute exercise (Marlin *et al*., 2002), some researchers reported that GSH release from the liver into blood for the use of other tissues and the GSH levels increased after exercise (Chiaradia *et al*., 1998). Observing such kind of contradictions arises from of the fact that the redox situation of GSH and change mechanism of its content in exercise not known well (Ji *et al*., 1993; Sen and Packer, 2000).

GSH, in muscles, is given to blood very fast while acute exercise occurs. Due to this, the level of blood GSH increases and the level of GSH in muscles and other tissues decrease (Ohkuwa *et al*., 1997;

Table 1: The concentration of lipid peroxidation (MDA, NO_2, NO_3), antioxidants (GSH, retinol, α-tocopherol) and vitamin D_3 in horses (n = 17)

Parameters	Before working (S±Sx)	After working (S±Sx)
MDA (nmol mL^{-1})	0.780±0.120	1.220±0.16*
Nitrate (NO_3) (ppm)	2.010±0.120	4.160±0.57*
Nitrite (NO_2) (ppm)	0.780±0.260	0.890±0.14*
GSH (mg dL^{-1})	10.610±0.680	20.850±0.67*
α-tocopherol (μg mL^{-1})	4.820±0.330	3.690±0.51*
Retinol (μg mL^{-1})	0.500±0.100	0.180±0.02*
Vit. D_3 (μg mL^{-1})	0.101±0.016	0.037±0.014*

*States the difference within the same row, p<0.05

Machefer *et al*., 2004). In this study, it was observed that the GSH level increased at whole blood after the horses work because the blood was taken in acute phase.

There are several indications that vitamin E level is one of the most effective antioxidant after exercise. The increase in plasma E level just after exercise was higher than other antioxidants in horse (Podolak *et al*., 2006). Besides, some researchers stated that the blood α-tocopherol level is lower in exercised horses than the unexercised horses. This decrease originated from the property of the vitamin E for cleaning free radicals and caused increase in the free oxygen radical production. The increase of vitamin E consumption during the acute exercise related to increasing of free radical levels (Kumar *et al*., 1992; Siciliano *et al*., 1997; Avellini *et al*., 1999). In this study, it's been observed that the level of α-tocopherol serum decreased in an important ratio after hard working.

Despite some studies stating that there is not a big change in serum vitamin A, after exercise, big differences between these levels, even significant decreases, have been reported in several studies by Kumar *et al*. (1992). In this study retinol level decreased in important ratio.

A metabolite of vitamin D is a hormone that regulates the metabolism of calcium and phosphorus. by Breidenbach *et al*. (1998), reported that, in contrast to other species, vitamin D does not appear to play a key role in regulating Ca and P (i) homeostasis in horses, however, D_3 level decreased in an important ratio after working in this study. This situation can be arised from the role of D_3 in calcium and phosphor metabolism. Increases in bone mineral density or cortical bone volume due to exercise by immature horses have been reported by several researchers. Subsequent aerobic exercises affect Ca and P serum concentrations, balance and digestibility in young, mature and aged horses. Physical exercise is known to affect calcium homeostasis in horses, may increase bone density and bone mass (Beunell *et al*., 1997; Vervuert *et al*., 2002). Furthermore, it is interesting to note that concentration of vitamin D_3 decreased after working as other lipid-soluble vitamins (retinol, α-tocopherol) decreased in this study.

In conclusion, in the present study a relationship was found between the physiological response to hard-working and some oxidant/antioxidant markers and vitamin D_3 in healthy-hard working horses.

In summary, it is observed that the level of MDA, NO_2 and NO_3 increased significantly ($p<0.05$) after working. While GSH concentration, increased after working; levels of retinol, α-tocopherol and vitamin D_3 levels decreased significantly ($p<0.05$). On the other hand, the vitamin D_3 levels were affected by hard working as other lipid soluble vitamins. There were a correlation between the physiological response to hard-working and some oxidant markers in healthy-hard working horses. These observations provide evidence that hard-working increases oxygen consumption and cause a disturbance of intracellular pro-oxidant -antioxidant homeostasis.

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