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Effect of exercise training, selenium and vitamin E on some free radical scavengers in horses (*Equus caballus*)

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Abstract

Physical exercise increases both tissue needs for oxygen and cellular respiration and causes an overproduction of free radicals. When free radical generation exceeds the cell's antioxidant capacity tissue-damage develops due to oxidative stress. Therefore, it appears important to increase the scavenger ability of the tissues. Controlled training and dietary supplements may provide ways of doing this. As a model, we used 3-year-old racehorses (*Equus caballus*) which underwent a series of different physical exercise trials before and after 70 days of daily training and dietary supplements (vitamin E and selenium). The above treatments were able to increase both red blood cell resistance to the peroxidative stress induced in vitro and the glutathione peroxidase activity in lymphocytes. Moreover, they were also able to decrease malondialdehyde (MDA) concentration in the plasma as well as vitamin E consumption and the mobilisation of low molecular weight antioxidants (total peroxyl-radical trapping) following the physical exercise trials. The results obtained indicated that the training and diet supplements we used were able to significantly increase horse antioxidant defences in both the extracellular fluids and blood cells of our horses, thus decreasing peroxidative phenomena following physical exercise. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Exercise; Free radicals; GSH-Px; Horse; Malondialdehyde; Peroxidative damage; Training and nutrition; TRAP; Vitamin E and selenium

1. Introduction

Under physiological conditions, the body usually has sufficient antioxidant reserves, such as superoxide dismutase, vitamin E and GSH-Px (glutathione: H₂O₂ oxidoreductase, EC 1.11.1.9), to cope with the production of free radicals [9,15,24]. However, when free radical generation exceeds the antioxidant capacity of cells or extracellular fluids, oxidative stress develops. Lipid peroxidation is one of the most important phenomena caused by free radicals, although protein and nucleic acid damage have been also described [6,10,18,19]. In turn the resulting modifications of these molecules cause tissue damage. Damage may be more serious in the skeletal muscles since they show low levels

of antioxidants compared with other tissues [35]. Furthermore, it has been suggested that the pathogenesis of exercise-induced myopathies and hemolysis in horses may be related to the modifications of cell membranes caused by lipid peroxidation and changes in antioxidative enzyme activities caused by free radicals [24,26,35]. Free radical overproduction in skeletal muscles may be caused by an increased demand for energy by the muscles caused by physical exercise. The increased demand for energy activates mitochondrial respiration and, as a consequence, increases oxygen uptake in the muscle. Finally, about 5% of the total amount of oxygen is converted into free radicals such as superoxide anion, hydrogen peroxide and hydroxyl groups [5,11,30].

Depletion of any of the antioxidant systems increases the vulnerability of various tissues and cellular components to reactive oxygen species (ROS), while tissues seem to increase their antioxidant defences under chronic activation [5,8]. Controlled long-term training may be one way of increasing antioxidant defences in the tissues of exercising subjects. [1,17,37].

Abbreviations: ABTS, 2,2'-azinobis(3-ethylbenzothiazoline 6 sulfonate); BHT, butylated hydroxytoluene; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; RBC, red blood cells; TRAP, Total peroxyl radical trapping ability.

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As reported for other animal species [37], we previously demonstrated that physical exercise in Maremmana stallions increases the consumption of natural antioxidants such as vitamin E and decreases the glutathione peroxidase activity in the blood [1,3]; furthermore, we also demonstrated the usefulness of dietary supplements with antioxidants (vitamin E and selenium) during training [3].

The aim of this study was to gain a better understanding regarding the effect of dietary supplements and a 70-day training period on the peroxidation phenomena induced by rigorous programmed physical exercise trials of increasing intensity. We compared malondialdehyde content (as end product of lipid peroxidation) [13] with blood vitamin E and low molecular weight antioxidants (TRAP) content [27,34] and with the enzymatic activity related to free radical scavenger systems in the plasma and blood cells of 3-year-old Maremmana stallions. We also assessed the resistance of the red blood cells to chemically induced oxidative stress and the activity and content (by ELISA test) in lymphocytes of GSH-Px whose importance in the cell's free radical scavenging capacity is well known [25]. In particular, regarding the last one, we set out to obtain some information regarding glutathione peroxidase activity and content in a cell which plays a role of primary importance in the inflammation phenomena which may be stimulated by physical exercise. Indeed, this enzyme is directly involved in redox modulation of a nuclear factor (NF- κ B) which plays an important role in the inducible transcription of several important immunologically genes [14].

2. Materials and methods

2.1. Animals

Eleven 3-year-old Maremmana stallions (*Equus Caballus*) from different horse farms were selected by genetic screening and clinically controlled to ascertain their aptitude to be utilised as stallions. The horses were placed on a farm in Tuscany, and after 20 days of rest and acclimation they underwent a special performance test. The performance test consisted of two sessions of a 200-m gallop at maximum speed (5 min of rest between the sessions), 18 h of rest, then a 3000-m gallop at maximum power. The test performed at the beginning of the study will be identified in the paper as 'first test' (T_I). After 70 days of training [1] and diet supplements, the horses underwent the identical performance test (T_{II}).

The training consisted of 30 min per day of physical aerobic exercise, 6 days a week. During the first week, exercise was without rider, exercise was then with a rider and its intensity was gradually increased [1,3].

The diet of the horses during the 70 days between the first and the second test was considered adequate for light work from a caloric standpoint (about 7 fodder units/day per horse, French's system, INRA [23]; one half of hay, one quarter of oats and one quarter of fodder) was supplemented with 20 μ g/kg per day of selenium and 40 mg/kg per day of vitamin E.

2.2. Samples

Blood samples were collected by jugular venipuncture in heparinised tubes before the exercise (T_0), immediately after the first bout session of gallop (T_1), after rest (T_3) and after a second bout of gallop (T_4). The samples were immediately centrifuged and the plasma was divided in three fractions for the different determinations. The fractions were stored under nitrogen at -30°C until analysis. The fraction for MDA determination was mixed with BHT to a final concentration of 0.04%. Erythrocytes were prepared as previously described [2]. Horse lymphocytes were isolated from buffy coats by centrifugation at $400 \times g$ for 30' at 20°C on a Ficoll–Paque[®] (Pharmacia Biotech, Italia, MI) density gradient. The blood mononuclear cells were recovered from the interface between the plasma and the Ficoll–Paque and subjected to a short washing step in phosphate buffered saline (PBS pH 7.6) to remove any platelets, Ficoll–Paque[®] and plasma. About 95% of the cells obtained were lymphocytes as determined by morphological criteria [15]. The lymphocytes were frozen at -30°C and then fragmented by thawing and sonication (47.6 KHz for 15'). The resulting lysates were used to measure the glutathione peroxidase content and its enzymatic activity.

2.3. Preparation of antibody against glutathione peroxidase

The antibody was prepared according to Bailey [4]. An antigen preparation of 250 μ g of GSH-Px (SIGMA) in 0.5 ml of H_2O and an equal volume of Freund's complete adjuvant (Sigma–Aldrich SrL, Milano, Italy) was injected subcutaneously into rabbit of the New Zealand strain. The immunisation was repeated three times by injecting 100 μ g of antigen emulsified with Freund's incomplete adjuvant (Sigma–Aldrich SrL, Milano, Italy). The rabbit was bled and the sera collected. The IgG was isolated by sodium sulfate precipitation (18% w/v). The IgG specificity was checked by using immuno-blotting [4,28].

2.4. Biochemical analyses

2.4.1. Vitamin E

The vitamin E extracted with *n*-esane from plasma was estimated by a reverse-phase HPLC method (Wa-

ters 510 pump; Spherisorb 5 μm ODS 250 \times 4.6 mm column; Waters 470 fluorescence detector λ_{exc} 298 nm and λ_{em} 328 nm) [32].

2.4.2. Malondialdehyde (MDA)

The MDA content in the plasma was assayed by separating the MDA/thiobarbituric acid adduct with a reverse-phase C18 Brownlee HPLC column 220 \times 4.6 mm (Waters 510 pump) and quantified by using fluorescence detector (Waters 470, λ_{exc} 515 nm and λ_{em} 550 nm) according to Draper et al. [12].

2.4.3. TRAP

We evaluated the total scavenger capacity of plasma by ABTS (Sigma) test according to Rice-Evans and Miller [31]. Briefly, the interaction of ABTS with the metamyoglobin- H_2O_2 derived radical species forms the radical cation of ABTS which has a characteristic absorbance at 734 nm. Antioxidants suppress the absorbance of the ABTS radical cation to an extent and on a time scale which are dependent on the their antioxidant capacity. The length of time of the lag phase (lag-time) before the reaction starts is proportional to the concentration of all the antioxidants in the sample.

2.4.4. GSH-Px activity assay

Enzymatic activity was measured according to Paglia and Valentine [29]. GSH-Px catalyses the reduction of hydroperoxides by glutathione; the glutathione disulfide production was monitored by coupling to the reaction catalysed by glutathione reductase. The rate of NADPH oxidation at 340 nm was used as an index of hydroperoxide reduction and the enzyme unit of GSH-Px was defined as micromoles of NADPH oxidised/min per mg total protein.

2.4.5. GSH-Px content (ELISA test)

The lymphocyte lysates (100 μg of the total protein made up in coating carbonate/bicarbonate buffer, pH 9.6) were attached in a non-specific manner to the solid phase (multiwell polystyrene assay plates). After an overnight incubation at 4°C, the wells were washed with 0.05% Tween 20 in PBS. An additional incubation with bovine serum albumin was carried out to reduce the background non-specific uptake. Rabbit anti-GSH-Px-IgG was then added and incubated to allow specific binding with lymphocyte GSH-Px. Finally, the antigen-antibody complex was quantified by the addition of horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma–Aldrich srl Italia, MI) and *o*-phenylenediamine was used as a substrate of peroxidase. The absorbance was determined at 490 nm in an automatic spectrophotometer (Fast Reader, Sorin Biomedica Italia). At the same time as the samples were tested, a series of known content samples (from 0.1 to

1 ng/ml) are also included. A standard curve was constructed from the absorbance of these and the unknown samples' content was calculated from that curve.

2.5. Oxidative stress in vitro

The erythrocytes underwent peroxidative stress by 2 mM (final concentration). *tert*-butyl-hydroperoxide incubation. BHT (0.1 mM final concentration) was added to interrupt the peroxidative chain reaction at fixed times (1, 2, 5 and 10 min); spectrophotometric measurements of the haemoglobin oxidative degradation products were calculated as described by Winterbourn [36] and expressed in % total haemoglobin.

2.6. Protein determination

Proteins of lymphocyte lysate were determined by the method of Lowry et al. [22].

2.7. Statistical analyses

The values are represented as Mean \pm SD. Significant differences between both pre- and post-diet supplementation and exercise values were computed by a three-way analysis of variance (PROC GML, SAS Software) using horses as an error term and by analysis of variance (one-way ANOVA) with Fisher and Scheffé post hoc tests (significant at 95%). The analysis of repeated measures ANOVA was also used to evaluate the significance of the RBC resistance to stress in vitro.

3. Results

Before training the MDA contents in horse plasma were significantly higher (Fig. 1), in resting conditions and after physical exercise, than those obtained after 70 days of training and diet supplement (three-way ANOVA: $P < 0.0001$). Both exercise trials increased the MDA content (three-way ANOVA $P < 0.05$). In the second test ($T_{3\text{II}}$) MDA was significantly lower than any value obtained in the first test.

Before the training period, the vitamin E content (Fig. 2) was very low at any time tested and no significant variations were found with exercise. In contrast, the vitamin E content at the beginning of the second test ($T_{0\text{II}}$) was about eight times higher than $T_{0\text{I}}$ (three-way ANOVA: $P < 0.0001$). It decreased significantly after the subsequent trials, reaching a value similar to that found during the first one ($T_{3\text{II}}$ as compared to $T_{3\text{I}}$). MDA and vitamin E contents were always significantly inversely correlated (linear regression: $P < 0.0001$).

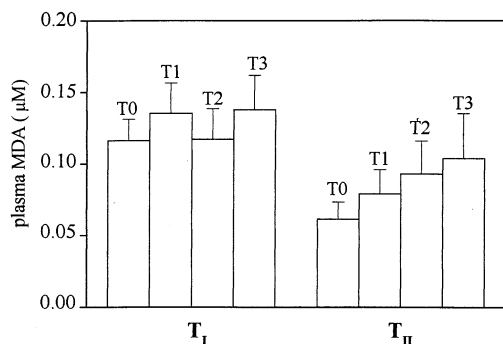


Fig. 1. The MDA content in plasma of the horses. On the left the results obtained during the first test (Roman numerals: T_I) and on the right those obtained during the second test (T_{II}). The ordinal numbers refer to when the blood samples were collected: before exercise (T_0), immediately after the first exercise (T_1), 18 h after resting (T_2) and after the maximum power test (T_3). The values are represented as mean \pm SD, $n = 11$. Significant results were computed by a three-way analysis of variance (PROC GML, SAS Software) using horses as an error term. T_I vs. T_{II} : $P < 0.0001$ (three-way ANOVA); T_{0I} vs. T_{3I} : $P < 0.05$ (three-way ANOVA); all times of T_{II} vs. each other: $P < 0.05$ (three-way ANOVA). For details see Sections 2 and 3.

The total antioxidant capacity of plasma was determined by measuring its 'TRAP'. In Fig. 3 we report the TRAP slopes: before the training the total antioxidant capacity of plasma increased significantly (three-way ANOVA, $P < 0.05$) along the entire series of trials (—■—), while no significant variations were found during the trials performed after the training (—●—).

The determinations of the resistance of RBC to the peroxidative stress induced in vitro before and after the 70 days of training and diet supplements were carried out only before and after the entire set of trials. Training and diet supplements profoundly modified RBC resistance to the peroxidative stress in vitro (repeated measures ANOVA: $P < 0.0001$). Indeed, the RBC resistance before the exercise deter-

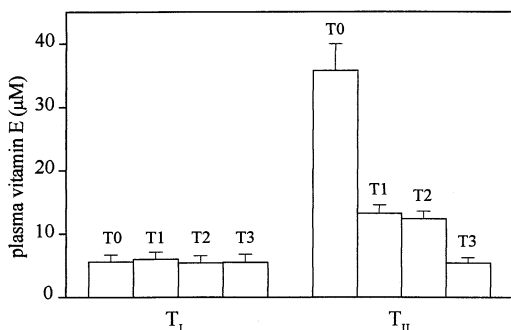


Fig. 2. Vitamin E content in the plasma of horses before (T_I) and after (T_{II}) the 70 days of training and diet supplements. During the first test no significant differences were found among the different times tested. T_{0I} vs. T_{0II} : $P < 0.0001$ (three-way ANOVA). T_{0II} vs. T_{1II} , T_{2II} , T_{3II} and T_{3II} vs. T_{1II} , T_{2II} : $P < 0.0001$ (three-way ANOVA). For details see Sections 2 and 3 and Fig. 1.

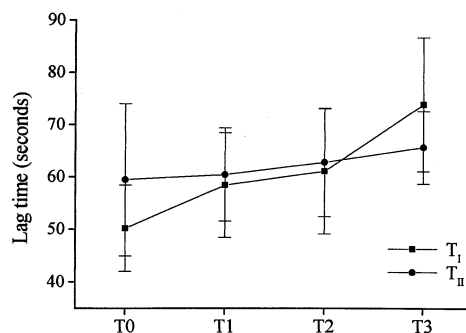


Fig. 3. Slopes of the TRAP determined in the plasma of horses during the first test (—■—) and the second test (—●—). During the second test no significant differences were found among the different times tested. T_{0I} vs. T_{1I} , T_{2I} and T_{3I} : $P < 0.05$ (three-way ANOVA). For details see Section 2 and Fig. 1.

mined at the time of the first test was even higher than that determined in the second test (Fig. 4a —■— as compared to Fig. 4b —□—). However, this resistance dramatically decreased after the set of trials performed during the first test (Fig. 4a, —■— as compared to —●—) while during the second test no differences in the RBC resistance were found before and after the physical exercise (Fig. 4b, —□— as compared to —○—).

The activity of the glutathione peroxidase in lymphocytes is reported in Fig. 5a while Fig. 5b shows the GSH-Px content determined by ELISA. The determinations were carried out only before and after the entire set of trials of physical exercise, i.e. after two sessions of a 200-m gallop at maximum speed, 18 h of rest, then a 3000-m gallop at maximum power, performed during the first and the second tests with the aim of evaluating the overall effect of physical exercise. At the beginning of the period of training and diet supplements the activity of GSH-Px was significantly lower than that determined after 70 days (three-way ANOVA: $P < 0.0002$). No significant modifications in enzyme activity were found after physical exercise (Fig. 5a, T_{0I} as compared to T_{3I} ; T_{0II} as compared to T_{3II}).

Fig. 5b indicates that no variation in enzyme protein content in lymphocytes took place as a consequence of exercise during the first test. After 70 days of training and diet supplements the enzyme protein content in lymphocytes appears increased (Fig. 5b, T_{0II} as compared to T_{0I} , ANOVA: $P < 0.01$, Scheffè post hoc test significant at 95%; three-way ANOVA, not significant). The apparent decrease in the proteins quantified by ELISA in lymphocytes after physical exercise was also of scarce statistical significance (Fig. 5b, T_{0II} as compared to T_{3II} , ANOVA: $P < 0.05$, Scheffè post hoc test significant at 95%; three-way ANOVA, not significant).

4. Discussion

Lipid peroxidation is a complex phenomenon that involves the generation of many products [19]. Among them MDA is one of the most significant end products of lipid peroxidation and its content, both in plasma and tissues, is generally accepted as an index of lipid peroxidation rate. The high content of MDA in the blood of our horses at the time of the first test may have been due to an accumulation of this molecule caused by previous oxidative stress (transport of the horse, new environment and so on) and suggests a low free radical scavenger capacity. In this regard, the lack of a clear physical exercise-dependent increase in MDA content does not seem surprising. In contrast, in the second test (70 days later) MDA content before the trials (Fig. 1, T_{0II}) was significantly lower compared with T_{0I} (Fig. 1). Moreover, MDA content progressively increased over the period of the different test trials, but never reached values obtained in the first test (Fig. 1, T_{3II} and T_{3I}). In our opinion, the MDA contents found and reported in Fig. 1 confirm that the physical exercise trials which we adopted caused an increase in free radical production. However, they also demonstrated that the training period and diet supplements adopted were able to significantly improve free radical scavenger systems (see the lower values of MDA content in the plasma found during the second test as compared to those of the first one in Fig. 1).

The higher content of vitamin E found after 70 days of diet supplementation, compared to that found before the treatment (Fig. 2, T_{0I} and T_{0II}), is not surprising as vitamin E concentration in the plasma reflects its con-

tent in the cells and is an index of its dietary intake [20]. Furthermore, since vitamin E is one of the most important free radical scavengers, the decrease of its content after the different trials during the second test (Fig. 2, T_{0II}, T_{1II}, T_{2II} and T_{3II}) indicates that the vitamin was consumed in order to counteract the lipid peroxidation induced by the free radical increase due to physical exercise [9,21]. This finding was further confirmed by the statistically significant correlation between vitamin E and MDA contents found in the serum of our horses (linear regression: $P < 0.0001$). In contrast, the data indicate that at the time of the first test the vitamin E content in the plasma of our horses was so low that no significant variations were found after the different test trials (Fig. 2, T_{0I}, T_{1I}, T_{2I} and T_{3I}). These results indicate that vitamin E content was probably at the minimum physiological level and was inadequate to counteract the peroxidation phenomena induced by physical exercise as confirmed by the high values of MDA contents.

The above findings seem to be further confirmed by TRAP slopes determined in the plasma during the two tests (Fig. 3). TRAP is an index of the scavenger capacity of the extracellular fluids and is determined by low-molecular weight antioxidants which act as chain-breaking scavengers in lipid peroxidation processes and as traps of peroxy radicals [31,34]. The increase in TRAP found in the horses before training and diet supplements (—■—) suggests that physical exercise caused a mobilisation of low molecular weight antioxidants necessary to support the inadequate efficiency or the presence of free radical scavenger systems while, in the presence of an adequate amount of scavengers such

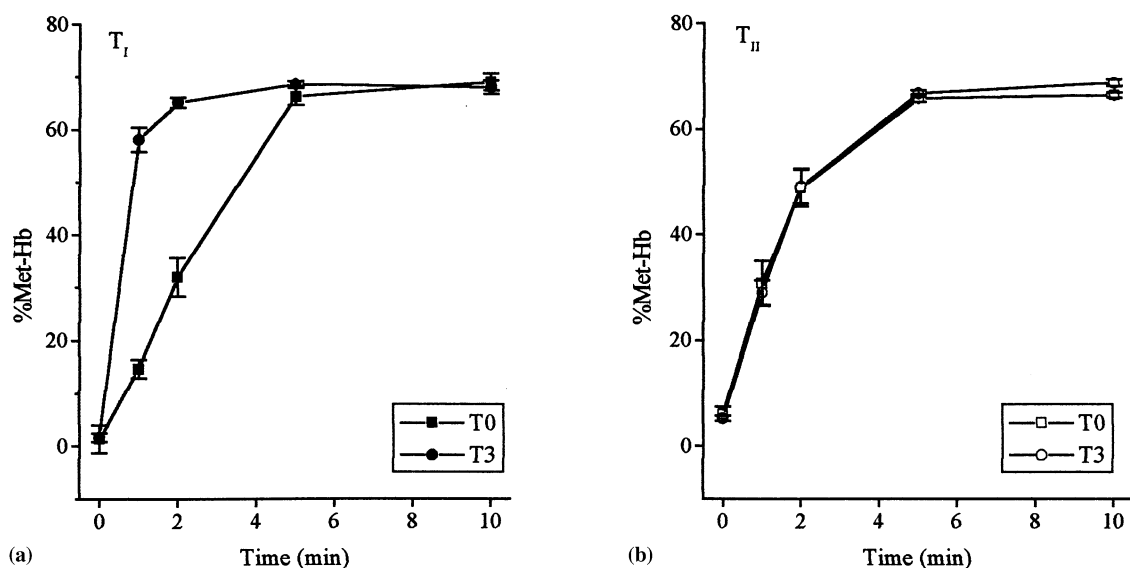


Fig. 4. RBC peroxidation stress in vitro induced by *tert*-butyl-hydroperoxide: slopes of the methaemoglobin determined in the horse erythrocytes during the first test (a) and the second test (b). The determinations were carried out only before (squares) and after (circles) the set of physical exercise trials. During the second test no differences were found before and after the physical exercise. T_{0I} vs. T_{0II}: $P < 0.0001$ (Repeated measures ANOVA); T_{0I} vs. T_{3I}: $P < 0.0001$ (Repeated measures ANOVA). For details see Section 2 and Fig. 1.

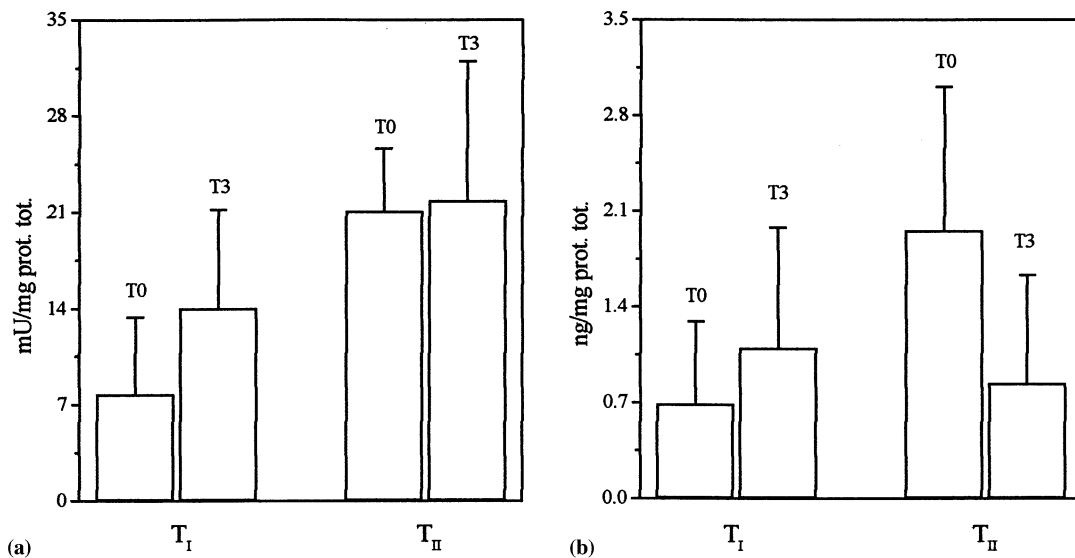


Fig. 5. a: GSH-Px activity (mU/mg of total protein) determined in horse lymphocytes during the first and second test, before and after the set of test trials. In both the tests (T_I and T_{II}) no significant modifications in enzyme activity were found after physical exercise. T_{0I} vs. T_{0II} ; $P < 0.0002$ (three-way ANOVA). b: ng of GSH-Px reactive to the ELISA test/mg of total protein determined in the lymphocytes at the same time as in a. T_{0II} vs. to T_{0I} , ANOVA $P < 0.01$, Scheffè post hoc test significant at 95%, three-way ANOVA, not significant. T_{0II} vs. to T_{3II} , ANOVA: $P < 0.05$, Scheffè post hoc test significant at 95% three-way ANOVA, not significant. For details see Section 2 and Fig. 1.

as vitamin E, the release of the above molecules (such as uric acid, tiols, bilirubin, ascorbic acid) from tissues [16] is unnecessary and the TRAP do not vary (—●—).

By summarising the results of MDA, vitamin E contents and TRAP in the plasma we may conclude that our horses presented a lower free radical scavenging capacity at the beginning of the experiment (T_I) than at the end (T_{II}). The scanty free radical scavenging capacity at T_I may be in part ascribed to a low content of vitamin E which, only partially balanced by low molecular weight antioxidants (TRAP), was unable to counteract the effects of a free radical increase caused by the physical exercise trials. Furthermore, the results presented in Figs. 1–3 indicate that the training and the diet supplements we used were able to significantly increase the free radical scavenging capacity as suggested by the decrease in peroxidation phenomena.

The improvement in the free radical scavenging capacity caused by the training and diet supplements was further confirmed by the results for red blood cell peroxidative stress in vitro (Fig. 4). Indeed, the RBC resistance to the peroxidative stress caused by *tert*-butyl-hydroperoxide may be considered as an index of the overall scavenging capacity of the cell [33]. As a consequence, the considerable modification of RBC resistance to the in vitro peroxidative stress found after the set of trials performed during the second test (Fig. 4, T_{II}) compared to that found after T_I , clearly indicates that the overall RBC scavenging capacity was significantly increased by training and

diet supplements. It is important to underline that the determinations of the resistance of red blood cells to the peroxidative stress induced in vitro before and after the 70 days of training, and diet supplements were carried out only before and after the entire set of trials: (1) to better evaluate the overall effect of physical exercise in RBC (Fig. 4a and b); and (2) because of the low significant modifications of erythrocyte resistance to the peroxidative stress in vitro found at T1 and T3.

Finally, the results for GSH-Px activity and content in lymphocytes (Fig. 5a and b) may be considered as an indication of the effect of training and diet supplements (mainly selenium enrichment). The 70-day period of training and diet supplements significantly increased GSH-Px activity (Fig. 5a, T_{0I} as compared to T_{0II}). This increased enzyme activity seems to be due to an increase in the enzyme content in the cells (Fig. 5b, T_{0I} as compared to T_{0II}) as reported in other animal species [7]. Unfortunately, the increase was statistically significant only with a less rigorous statistical method (ANOVA $P < 0.01$, Scheffè post hoc test significant at 95%, but three-way ANOVA was insignificant, $P = 0.0696$).

In conclusion, the results indicate that the training and diet supplements increased antioxidant defences in extracellular fluids and blood cells of our horses. In turn, this increased free radical scavenger capacity reduced the free radical increase following physical exercise, thus efficiently counteracting lipid peroxidation. As a consequence, the risk of cell damage was reduced and, for horses, exercise-induced myopathies

and hemolysis linked to this phenomenon. Furthermore, another important conclusion is that the diet might be inadequate regarding vitamin E and selenium for animals that are about to undergo a race or trials involving acute strenuous and/or sustained physical exercise. Therefore, we suggest that some of the above blood tests should be added to other routine tests to better establish the nutritional status as well as the training level of the animals which will undergo physical exercise.

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