Effects of an aerobic training program on oxidative stress biomarkers in bulls

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ABSTRACT: The aim of this study was to evaluate the effect of aerobic training (16 weeks – T1 and 24 weeks – T2) on oxidative stress biomarkers. To this end, GSH, GSH-peroxidase (GSH-Px) and catalase (CAT) activity were analysed in plasma. Nine bulls (3–4 years), were included in this work. The exercise training protocol was performed in a track ("taurodromo") three days per week for 24 weeks and consisted of 400 m warming up, 1200 m to 4–5 m/s, two minutes' resting, 1200 m to 4-5 m/s and, finally, 400 m walking. The results reflected that GSH-Px activity was higher at T1 (6.18 ± 0.45) than at baseline (T0; 2.31 ± 0.08) while the GSH level (2.98 ± 0.37) was lower vs. T0 (14.59 ± 3.40). Moreover, there were significant increases in GSH-Px (18.23 ± 1.36) and CAT (2.52 ± 0.04) activities and the recovery of basal values in GSH (11.75 ± 2.84) in T2. In conclusion, the type of training carried out in this study involved two well-defined stages: (i) a period of perturbation, followed by (ii) adaptation. The former stage was characterised by the induction of oxidative stress manifested as a decrease in the GSH, and the latter (T2) by the recovery of this non-enzymatic antioxidant.

Keywords: aerobic training; bulls; glutathione peroxidase; oxidative stress; reduced glutathione

The generation of reactive oxygen species (ROS) is a perpetual biological process in eukaryotic cells. Oxidative stress has been defined as an imbalance between oxidants/antioxidants, prompting damage and death (Halliwell and Gutteridge, 1989; Sies, 1991; Kirschvink et al., 2008). Organisms are constantly exposed to exogenous and endogenous ROS and reactive nitrogen species (RNS) such as nitric oxide and superoxide anions (Moslen, 1994; Niviere and Fontecave, 1995; Kowaltowski and Verseci, 1999; Kirschvink et al., 2008). To block the injurious effects of ROS and RNS, organisms have evolved different antioxidative systems (enzymatic and non-enzymatic) (Cheeseman and Slater, 1993; Kirschvink et al., 2008).

Exercise is associated with increases in both ATP requirements and aerobic and/or anaerobic metabolism, which result in higher levels of ROS and RNS (Davis et al., 1982; Jackson et al., 1985; Viguie et al., 1993; Inoue et al., 1993; Sen, 1995; Nojima et al., 2008). Thus, when exercise is strenuous it causes oxidative stress and cell damage, but when done in moderation, it increases the expression of antioxidant enzymes (Gomez-Cabrera et al., 2008). The preventive effect of regular exercise is, at least partly, due to oxidative stress-induced adaptation. This response is systemic and includes enhancement of antioxidant systems and the reduction of oxidative damage due to changes in redox homeostasis (Radak et al., 2008). On the other hand, although training increases the antioxidant defence system of the organism, prolonged periods of training may cause disturbances in the oxidant/antioxidant equilibrium (Avellini et al., 1995; De Moffarts et al., 2005; Kirschvink et al., 2008).

Additionally, a relationship has been suggested between muscle problems and disturbances in muscle homeostasis, fatigue and injury with oxidative stress induced by exercise (Powers et al., 1999). With this information in mind, it can be assumed that problems with muscular weakness are associated with the presence of oxidative stress. However, although studies in other animal species relate exercise and oxidative damage, its mechanisms and pathways are not clear.

In late-pregnant and non-pregnant cows training for 60 days improved physical fitness (Davidson and Beede, 2009), but oxidative stress was not evaluated. The latter has only been determined in cows during pregnancy, lactation, milk production or the administration of certain substances (Castillo et al., 2005; Abd Ellah et al., 2009; Pedernera et al., 2009; Albera and Kankofer, 2010; Antoncic-Svetina et al., 2010). However, none of these studies in cows associate exercise with oxidative stress.

The main aim of this study was to evaluate in bulls the effect of an aerobic training program (16 weeks and 24 weeks) on oxidative stress biomarkers. To this end, oxidative stress biomarkers such as reduced glutathione (GSH), GSH-peroxidase (GSH-Px) and catalase (CAT) activity were analysed in plasma.

MATERIAL AND METHODS

Animals and training

Nine bulls of the "*Bos taurus ibericus"* breed, aged between three and four years, clinically healthy and free of medication were included in this work. They were studied at a baseline situation and during an aerobic training program. The bulls were kept outdoors in an extensive sedentary (stationary) regime, with no housing and food (mixed feeding) and water *ad libitum*. All procedures regarding animal welfare were in accordance with the European Community Council Directive of 24 November 1986 (86/609/ECC) and RD 223/1988, and were approved by the University of Cordoba (Spain) Bioethics Committee.

The exercise training protocol was carried out on a 1600 m track (Taurodromo) three days per week for 24 weeks. Tests were performed on each bull at the beginning of the training protocol and over the course of the whole study. The protocol consisted of 400 m warming up at a slow pace, 1200 m to 4–5 m/s (speed controlled with a timer, Sportline Walking Advantage 228 Giant Stopwatch®), two minutes' resting, 1200 m to 4-5 m/s and, finally, 400 m walking (modified protocol of Davie et al., 2002).

Sample collection

To evaluate the effect of the training program, samples were collected one week before beginning the aerobic training program (baseline – T0; at the end of October); and 16 (T1; at the beginning of March) and 24 weeks (T2; at the beginning of May) after aerobic training. Before training, the bulls did not do any exercise whatsoever.

Venous blood samples were collected when animals were immobilized (Large Animal Immobilon®). Blood samples were taken inside 3–4 min after immobilization from *vena caudalis mediana* and placed in collection tubes (Vacutainer, Becton-Dickinson) with double ended needles, allowing fast, easy sample collection. These were placed in heparin-lithium tubes (for biochemical and oxidative stress analysis) (Tapval®, Barcelona, Spain). The heparin-lithium tubes were centrifuged at 2500 rpm for 10 min at 4 °C, and plasma was aliquoted and stored at –40 °C before being assayed.

The following parameters were analysed in sample blood: number of red blood cells (RBC; $\times 10^6$ cells/mm^3), hematocrit value (%) and hemoglobin (g/dl). All the assays were carried out using a semi-automatic cell counter (Sysmex F-820, Sysmex Europe GMBH). The following were evaluated in plasma: (i) biochemical parameters: creatin kinase (CK, IU/l), aspartate aminetransferase (AST, IU/l), alkaline phosphatase (AP, IU/l), lactate (mmol/l), uric acid (mg/dl) and glucose (mg/dl), the parameters being quantified by kits purchased from Atom, S.A., Barcelona, Spain (i.e. BioSystems®); and (ii) oxidative stress biomarkers: the GSH levels (nmol/mg protein) were measured using reagents purchased from Oxis International (Portland, OR, USA), i.e., GSH-420 kit (Figure 1), whereas glutathione peroxidase (GSH-Px, IU/mg protein) and catalase (CAT, (IU/mg protein) × 100) activities were evaluated by the Flohe and Gunzler (1984) and Aebi (1984) methods, respectively. Finally, protein concentrations were determined by the Bradford method (1976), using bovine serum albumin as a standard.

Although the GSH levels and antioxidant enzyme activities could be measured in red blood cells, their measurement in plasma provides an approximation of the blood state, reflecting changes at the intracellular level (Jones et al., 2000; Tunez et al., 2007).

Statistical analysis

A statistical analysis of data was made by means of the Sigma Plot® statistical software package.

Table 1. Effects of aerobic training program on blood parameters; mean ± SEM; n = 9 animals

	T0	T1	T2
RBC (× 10^6/mm^3)	7.00 ± 0.36	7.62 ± 0.36	9.15 ± 0.55^{++}
Hemoglobin (g/dl)	12.51 ± 0.53	14.27 ± 0.41*	13.60 ± 0.38
Hematocrit (%)	40.58 ± 1.64	48.66 ± 1.80	57.88 ± 2.11^{+}
MCV (fl)	58.40 ± 1.71	64.80 ± 1.46*	67.19 ± 1.46
MCH (pg)	18.02 ± 0.62	17.68 ± 1.19	15.17 ± 0.74

Significance levels: *P < 0.05 vs T0; ^{+}P < 0.05 vs T1; ^{++}P < 0.01 vs T1

The Shapiro-Wilk test did not show any significant departure from normality in the distribution of variance values. To evaluate variations in data, a one-way analysis of variance (one-way ANOVA) was corrected with the Tukey test. The level of statistical significance was set at P < 0.05. All results are expressed as mean ± SEM.

RESULTS

Effect of training program on blood biochemical parameters

The training induced significant increases in plasma glucose and AP levels in T1 compared with T0 (P < 0.05 and P < 0.001, respectively; Table 2). Additionally, in these training times an enhancement of the haemoglobin concentration was observed (P < 0.05; Table 1). On the other hand, in T2, as low lactate levels only have physiological significance when values increase by 2 or 4 mmol/l, a significant decrease in lactate levels in relationship with T1 (P < 0.05) was found, whereas RBC and hematocrit were higher in T2 than in T1 (P < 0.01 and P < 0.05, respectively; Table 1).

Effect of aerobic training on oxidative stress biomarkers

The GSH levels were lower in T1 than in T0 (P < 0.01; Figure 1). Additionally, Table 3 presents the values of antioxidative enzyme activities in nine bulls. GSH-Px activity was higher in T1 than in T0 (P < 0.01), whereas CAT® did not show any statistically significant changes. Moreover, there were significant increases in GSH-Px and CAT activities in T2 when compared to T1 (P <0.001), whereas GSH showed an important 294% enhancement with respect to T1, although it was not statistically significant (P = 0.06).

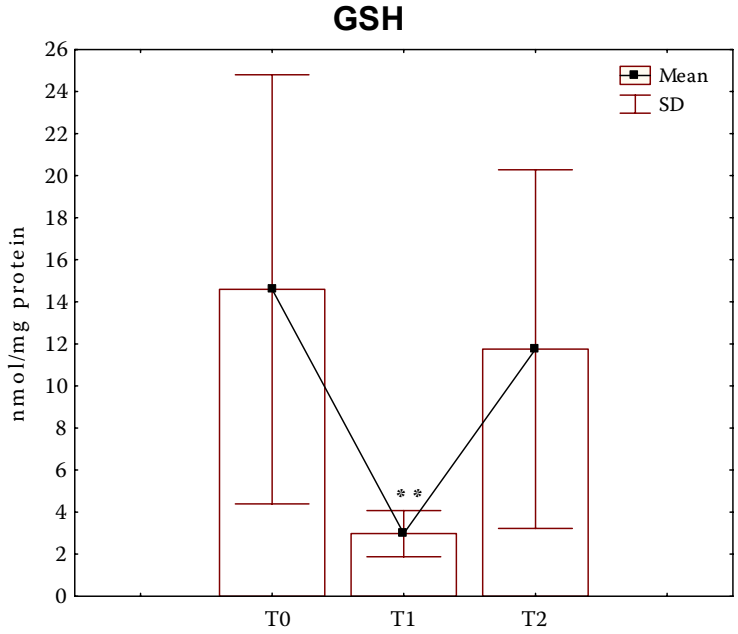

Figure 1. Effect of aerobic training program on GSH; mean ± SD, n = 9 animals

**P < 0.01 vs T0

Table 2. Effect of aerobic training program on biochemical parameters; mean ± SEM; n = 9 animals

	T0	T1	T2
CK (IU/l))	492.70 ± 71.84	408.09 ± 50.79	359.10 ±74.57
AST (IU/l)	154.42 ± 12.06	201.99 ± 23.50	164.34 ± 12.18
AP (IU/l)	128.51 ± 10.04	196.82 ± 14.27***	181.21 ± 14.71
LDH (IU/l)	1213.95 ± 204.55	631.50 ± 37.53*	1401.33 ± 12.80^{+}
Lactate (mmol/l)	2.68 ± 0.32	3.69 ± 0.41	1.87 ± 0.27^{+}
Uric acid (mg/dl)	1.09 ± 0.23	0.99 ± 0.06	0.90 ± 0.10
Urea (mg/dl)	39.01 ± 6.87	48.45 ± 1.42	30.50 ± 2.67^{+}
Creatinine (mg/dl)	1.74 ± 0.21	2.23 ± 0.15	1.34 ± 0.10^{++}
Glucose (mg/dl)	113.62 ± 7.71	176.63 ± 18.80*	165.37 ± 12.17
Total protein (g/l)	8.02 ± 0.22	7.23 ± 0.13*	8.20 ± 0.12^{+}

Significance levels: *P < 0.05 vs T0; ***P < 0.001 vs T0; ^{+}P < 0.05 vs T1

Table 3. Effect of aerobic training program on oxidative stress biomarkers; mean ± SEM; n = 9 animals

	T0	T1	T2
GSH (nmol/mg protein)	14.59 ± 3.40	2.98 ± 0.37**	11.75 ± 2.84
CAT (IU/mg protein) × 100	0.78 ± 0.18	0.88 ± 0.02	2.52 ± 0.04^{+++}
GSH-Px (IU/mg protein)	2.31 ± 0.08	6.18 ± 0.45**	18.23 ± 1.36^{+++}

Significance levels: **P < 0.01 vs T0; ^{+++}P < 0.001 vs T1

DISCUSSION AND CONCLUSIONS

The impact of exercise and training on the oxidant/antioxidant balance has been widely investigated in laboratory rodents, in humans and in horses (Sen and Packer, 2000; Leeuwenburgh and Heinecke, 2001; Kirschwink et al., 2008). However, the effect of training on oxidative stress in non-athletic species, e.g., bulls, as an integral part of their welfare, has never been studied. This point constitutes the salient novel feature of this study.

The results of this study showed: (i) that there was a period of imbalance in T1; and (ii) an adaptation-compensation period in T2.

The imbalance found in T1 involved the generation of oxidative stress characterized by a diminution in the GSH (Figure 1) and a concomitant rise in GSH-Px activity (Table 3), together with significant increases in Hb (Table 1), AP, glucose levels and non-significant ones in lactate (Table 2). These data agree with studies indicating that there is a period during training which is characterized by changes in oxidative stress markers (Gomez-Cabrera et al., 2008; Nojima et al., 2008). Thus, De Moffarts et al. (2004) observed increases in GSH-Px and SOD activity in Standardbred horses after twelve weeks of aerobic and anaerobic training, while Oztasan et al. (2004) found SOD and GSH-Px increases in rats after eight weeks of training. Other studies have shown a decrease in GSH with training and no change for GSH-Px in horses (Willians and Carlucci, 2006) .The differences found between our study of 16–24 weeks and the sources cited above could be due, at least partly, to three factors: (i) the animal studied; (ii) the type of exercise applied in the training; and (iii) the monitoring protocol established (0, 16 and 24 weeks).

A probable explanation for the imbalances found in oxidative stress markers in the T1 period might be the role played by hydroperoxides. The latter are eliminated by the action of GSH-Px (Kirschvink et al.2008), an enzyme which uses GSH to reduce water-producing hydroperoxides and GSSG (an oxidized form of glutathione). This situation would explain and link the increase in GSH-Px activity to the fall in GSH. On these lines, Oztasan et al. (2004) demonstrated a compensatory increase in

GSH-Px in sedentary rats after exercise to the increase in GSSG (glutathione in its oxidized form) by glutathione oxidation due to increased reactive oxygen species in blood during acute, exhausting exercise. Meister and Anderson (1983), Kretschmar and Muller (1993), Ji (1995), Somani et al. (1995), Ji and Leeuwenburgh (1996) and Powers et al. (1999) have related the decreases in GSH to the existence of oxidative damage.

As mentioned before, increases in the AP, glucose and Hb levels (Tables 1 and 2) were in parallel to the oxidative stress. In this direction, there are studies showing an increase in Hb resulting from erythrocyte destruction caused by exercise (Szygula, 1990; Weight et al., 1991; Smith, 1995), which is partly responsible for a higher production of ROS, and the consequent oxidative stress in T1. This released hemoglobin is oxidized to metahemoglobin, producing ROS, which favours lipid peroxidation and the formation of hydroperoxides (Cooper et al., 2002; Murakami and Mawatari, 2003; Bonilla et al., 2005). Besides this, AP and glucose levels were within physiological ranges.

Also, our results show that after the imbalance period (T1), there was a period of response, and, possibly, of adaptation (T2) characterized by an increase in non-enzymatic antioxidant defenses, evaluated by means of the peripheral levels of GSH, together with higher CAT and GSH-Px enzymatic activity. The increase in GSH (Figure 1), observed in many other studies (Ji et al., 1991; Ji and Fu, 1992; Ji et al., 1992; Somani et al., 1995; Elosua et al., 2003) can be explained as an adaptation-compensation response to an increase in ROS production. In turn, the latter were increased as a result of a greater consumption of oxygen by the electronic transport chain during the training. This situation explains the greater GSH-Px and CAT activity (Table 3), if it is borne in mind that the increase in O_2^- proceeding from the electron transport chain would be blocked by the SOD producing glutathione peroxidase These data are in agreement with previous studies which reported increases in GSH-Px and CAT after aerobic training periods (Ji, 1999; Elosua et al., 2003). According to Powers et al. (1999) increased concentrations of GSH, GSH-Px and CAT reduce the risk of cell injury, improve performance, and delay muscle fatigue. On the other hand, the biochemical and hematic parameters affected in T1 tend to become normalized (Tables 1 and 2). Transient changes in hematocrit, RBC and total protein in T2 were suggestive of possible fluid changes in plasma volume due to the high temperature in the month of May during the collecting of samples (32–35 °C).

Taken as a whole, these data indicate that, as exercise becomes regular, a greater consumption of O_2 is triggered in order to produce energy, with the consequent production of ROS and the adaptation of antioxidant systems to prevent oxidative damage.

In brief, the type of training carried out in this study involves two well-defined stages of oxidative stress: (i) is the induction of significant oxidative stress characterized by a decrease in GSH, and, in (ii) (evaluated at 24 weeks), the recovery of this non-enzymatic antioxidant. In spite of these results, more studies would be necessary to assess and tease out the molecular mechanisms involved in the adaptation to exercise.

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