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Changes in the redox status and inflammatory response in handball players during one-year of competition and training

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Changes in the redox status and inflammatory response in handball players during one-year of competition and training

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Abstract

The present research was designed to evaluate the adaptive responses to oxidative stress and inflammation in handball players subjected to well-controlled training intervals over one-year of competition. Seven blood samples were collected over the season of the study, approximately one a month. Plasma lipid peroxidation, nitrite, cytokines (IL-1β, IL-6, INFγ and TNFα), and the glutathione cycle in erythrocytes, were measured. Exercise intensity, measured with the Borg's scale, increased significantly up to the middle of the competition season, coinciding with maximal creatine kinase and lactate dehydrogenase values, and then decreased at the end of the study. The inflammatory markers including nitrite, IL-1β, IL-6, and, to a lesser extent INFγ, increased early in the training season, and remained elevated until the end of the study. TNFα, however, remained low during the season. The oxidative stress response included a transient increase of the glutathione disulphide/glutathione ratio and glutathione reductase activity at the beginning of the study, returning to basal values somewhat later. Glutathione peroxidase also increased at the end of the training season, and lipid peroxidation levels remained low during the athletic season. These results suggest that well-trained athletes were best adapted to the oxidative response, although the beneficial effects of some of the inflammatory cytokines on skeletal muscle myogenesis and repair cannot be ruled out.

Keywords: *exercise, cytokines, antioxidative defence, skeletal muscle*

Introduction

The generation of reactive oxygen and nitrogen species is a physiological process that follows the normal cell metabolism. These reactive oxygen species and reactive nitrogen species are controlled by the endogenous antioxidant system. However, the overproduction of such molecules may be harmful because most of them are free radicals able to oxidise basic cell structures such as lipids, proteins, and DNA, a process known as oxidative stress (Kyparos, Vrabas, Nikolaidis, Riganas, & Kouretas 2009). Oxidative stress refers to the imbalance between pro-oxidant production and antioxidant defences, in favour of pro-oxidants generation (Fisher-Wellman & Bloomer, 2009).

Physiologically, reactive oxygen and nitrogen species are regulatory molecules controlling a variety of processes including gene expression, protein turnover, inflammation, and cellular differentiation (Ji, Gomez-Cabrera, & Vina, 2006). The induction of reactive oxygen and nitrogen species by physical exercise is well documented in both animal and human studies (Oostenbrug, Mensink, Bar, & Hornstra, 1997). In turn, reactive oxygen and nitrogen species are released from muscle, endothelial, and immune cells to favour the body's adaptation to intense physical exercise. This adaptation includes changes in the immune and energetic status related to cytokine synthesis such as tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) (Zembron-Lacny et al., 2010).

Exercise increases oxygen consumption particularly in the skeletal muscle, compared with the resting state. Increased oxygen consumption during exercise can increase free radical activity (Martarelli

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& Pompei, 2009). Therefore, physical activities such as running, cycling, and swimming may cause free-radical production both in humans and experimental animals (Escames et al., 2011). However, exercise-induced free radical production is lower during low-intensity exercise than with intense exercise, and less free radical damage is observed during the former (Pepe, Balci, Revan, Akalin, & Kurtoglu, 2009). Moreover, well-tolerated endurance training, with intensities 60% below the maximal oxygen consumption (VO_2max), enhances blood antioxidant capacities at rest as well as during exercise, presumably due to oxidative stress exposure (Brites et al., 1999). By contrast, strenuous exercise of long duration and exhaustive sprint training may overwhelm the body's capacity to detoxify reactive oxygen and nitrogen species from the body. It is now clear that oxidative stress, muscle damage, and inflammation are associated events during exercise of high intensity (Serrano et al., 2010).

Skeletal muscle has recently been identified as a source of cytokines, now called myokines (Pedersen, 2011). Given that skeletal muscle is the largest organ in the human body, the discovery that contracting skeletal muscle secretes proteins sets a novel paradigm: skeletal muscle is an endocrine organ producing and releasing myokines in response to contraction, which can influence metabolic activity in other tissues and organs. Thus, a possible link between skeletal muscle contractile activity and immune changes has been established (Pedersen & Febbraio, 2008). Among other cytokines, exercise-induced plasma IL-6 concentrations increase in an almost exponential manner, peaking at the end of the exercise or shortly thereafter, and followed by a rapid decrease towards pre-exercise levels (Sacheck et al., 2006). Because IL-6 is a classical inflammatory cytokine, it was initially thought that the IL-6 response was related to muscle damage. It has become evident, however, that muscle damage is not directly related to increase IL-6 during exercise (Pedersen, 2011).

A feature of handball sport is the intermittent activity, since the player does not participate in a continuous game. Instead handball players run, walk, jump, dribble, strike, etc., so there is a discontinuity in their efforts and, thus, their metabolic demands encompass both aerobic and anaerobic energy pathways (Wallace, Mills, & Browning, 1997). This means that the training of the players should be designed to adapt the organism to these variable energy demands, which may have different impacts on reactive oxygen and nitrogen species generation and damage. There is, however, controversy in the literature; some authors report a significant increase of oxidative stress and reduction in the antioxidant system during intensive training (Finaud et al., 2006), other authors report a beneficial effect of training in the adaptation to the redox response to exercise (Schippinger et al., 2009; Teixeira et al., 2009). Thus, training strategy may be critical in the adaptation of the athlete to oxidative stress. If a training design is able to accomplish this objective, physical performance should improve. Consequently, this study was designed to clarify the influence of a well-controlled training plus competition schedule during one-year of competition and training on oxidative stress and inflammation in professional handball players of the Spanish Handball League.

Methods

Participants

Sixteen professional players of the Spanish Handball League (age, 22.7 ± 3.1 years; height, 187 ± 5.6 cm, and weight, 86.8 ± 5.5 kg; body mass index [BMI], 24.57 ± 2.66) were enrolled. Informed consent was obtained from all participants in the study, which was approved by the Ethics Committee of Granada's University and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All participants were highly trained and performed regular exercise training, on a daily basis. To estimate average energy and nutritional intake, participants recorded their dietary intake. A trained nutritionist gave them detailed verbal instructions about proper dietary recording. A full description of foods and fluids consumed was requested, including the brand names of packaged food, cooking or processing methods, and food items and ingredients added during preparation. Participants estimated the amount of food or fluids consumed by referring to the weight or volume information provided on food package or by using standardised household measures. Dietary-record information was converted to energy and nutrients with the software DIAL (Alce Ingenieria, Madrid, Spain). This program was supplemented with information for composite dishes, commercial foods, and sports foods whenever reliable nutritional composition data could be obtained. Diet was monitored to yield the following nutritional profile of the players (mean ± standard deviation): proteins, 142.3 ± 29.6 g (17%); fat, 126.7 ± 31.2 g (34%); carbohydrates, 399.7 ± 78.4 g (49%); total Kcal, 3300.

Training design

The participants were studied from the third week of August to the fourth week of April, the period corresponding to the Spanish handball league. The training plan was based on Seirul-lo and Verjoshansky's model (Issurin, 2010), and it was structured in macrocycles - mesocycles - microcycles

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- sessions. The macrocycle is the largest unit of time that includes 4.5 months; every month corresponds to one mesocycle, and each mesocycle is divided into four week-long microcycles, yielding a total of 36 microcycles (Table I). The full training plus competition period was divided into macrocycles I and II. The training sessions included five sessions per week from 20:30 to 23:30 h (Monday to Friday), and one handball match on Saturday, according to the competition schedule (Table I).

The cycles of training and competition season were scheduled as follows: 1) macrocycle I: containing a first microcycle of gradual training, with a high load volume (150–200%) and low-medium intensity (30–50%). This starting microcycle was followed by an additional high-intensity microcycle (shock load), reaching 100% of the maximal intensity with a low load volume (50%); this type of microcycle was repeated one week later. The additional 13 training microcycles, were designed to progressively increase the exercise intensity, reaching the maximal value at microcycle 16 (Figure 1). The load applied during these microcycles was near to 80% maximal intensity, with a load volume of 60–70%. Thus, the objectives of this macrocycle were to attain aerobic endurance, maximal aerobic capacity, and maximal muscle strength. Macrocycle I finished with two microcycles of physical maintenance, with learning tasks to improve the abilities of the players, including team spirit and team play. The intensity applied in this period did not exceed from 80% of maximal load; 2) macrocycle II: starts with 1 microcycle of tapering and 6 microcycles of physical maintenance, with similar characteristics as described for macrocycle I. After that, there were 11 microcycles mainly characterised by 80% maximal intensity and 60–70% volume, and learning. The objectives of this second macrocycle, with high intensity and moderate volume, were to maintain maximal aerobic power, competition velocity, and to attain the best team play. The objectives of this type of microcycle were that the participants could adapt to the loads to which they were subjected, while at the same time, and from a psychological point of view, they moved away from the competition stress. Additionally, recovery and control microcycles were placed along macrocycles I and II, to evaluate the participants of the study. For this reason, the load volume in recovery and control microcycles is reduced to 30% and only physical and technical tests are performed. This information is used for analysing the progression of their conditional and coordination capacities.

Exercise intensity measurement

The scale of rate of perceived exertion (RPE) method was used to quantify the exercise intensity developed by the players (Borg & Dahlstrom, 1962). The RPE scale is commonly used in either team or individual sports, and it ranges the exercise intensity from 6 to 20 points. A given value of this scale correlates well with the heart rate of the athlete. Using this scale, the exercise intensity of the players enrolled in the study was monitored (Figure 1). A value of 6 corresponds to no exertion at all, 7 is extremely light, 9 is very light, 11 is light, 13 is somewhat hard, 15 is hard, 17 is very hard, 19 is extremely hard and 20 is maximal exertion.

Samples

Blood samples were taken during the competition season (Table I). The first blood sample (Pre) was taken on the first day of the training just before the training started, and the last one once the competition finished, at rest (Post). During this period of rest, the participants were not subjected to controlled training, and they were free to perform any physical activity unrelated to competition, i.e., low intensity activity. The remainder of the samples were always obtained within the recovery and control microcycles, at different times along the competition season (Table I). Fasting blood samples were collected from the antecubital vein at 08:00 h and centrifuged at 3,000 *g* for 10 min at 4°C. Aliquots of plasma were frozen at –80°C, and the erythrocyte fraction was washed with cold saline thrice and frozen at –80°C.

Plasma lipid peroxidation assay

Plasma samples were thawed and centrifuged at 5,000 *g* for 5 min, and 200 μl of the supernatants were used for lipid peroxidation measurement. The oxidation of polyunsaturated fatty acids yields lipid peroxides, which are unstable and decompose to reactive carbonyl compounds including malondialdehyde and 4-hydroxyalkenals. Measurement of these carbonyls is widely used as an index of lipid peroxidation (Esterbauer & Cheeseman, 1990), which was measured with a commercial kit (Bioxytech lipid peroxidation-568 assay kit, OxisResearch, Portland, OR, USA). The concentration of lipid peroxidation was assessed at 568 nm, and expressed in $\mu mol \cdot l^{-1}$.

Measurement of glutathione and glutathione disulphide

Washed red blood cells were haemolysed in phosphate buffer (10 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid disodium salt, pH 6.25), desproteinised with ice-cold 10% trichloroacetic acid, and centrifuged at 20,000 *g* for 15 min. Supernatants were used for

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Table I. Distribution of macrocycles, mesocycles and microcycles during the training plus competition season.

MACROCYCLE 1 (20/08/2008 to 04/01/2009)

MESOCYCLES (MONTHS)	18/08 to 14/09				15/09 to 12/10				10/13 to 11/09				10/11 to 07/12			
MICROCYCLES (WEEKS)	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16
←Type McC/Ss	GR	S	TL	SM	TLM	TLM	TLM	RC	TLM	TLM	RC	TL	TLM	TLM	TLM	RC
←Ss/Week	3	8	9	12	8	6	6	4	6	6	4	5	6	6	6	4
←h' Ss/Week	6	24	28	28	20	18	18	8	16	16	8	12	14	16	14	8

MACROCYCLE 2 (05/01/2009 to 26/04/2009)

MESOCYCLES (MONTHS)	08/12 to 04/01				05/01 to 01/02				02/02 to 22/02			23/02 to 15/03			16/03 to 05/04			06/04 to 26/04		
MICROCYCLES (WEEKS)	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
←Type McC/Ss	AM	AM	T	AM	AM	AM	AM	RC	AM	TLM	TLM	MR	AM	AM	AM	RC	AM	TLR	AM	AM
←Ss/Week	5	5	3	5	5	5	5	4	5	6	6	4	5	5	5	3	5	6	5	5
←h' Ss/Week	12	12	6	12	14	15	14	10	12	14	16	10	14	15	15	8	14	12	12	12

REST (08/10/2009)

INTENSITY EXERCISE SESSIONS

Weeks 01–16	Weeks 17–25	Weeks 26–36
initial gradual training + progressive high-intensity training	physical maintenance (apprenticeship training programme)	moderate-high training + apprenticeship training programme

100%

50%

30%

DATES OF SAMPLING (MONTH/DAY)

Pre	08/10	29/10	03/12	28/01	26/03	Post

Bottom: the shadowed area reflects the changes in exercise intensity during the study in terms of % of the maximum intensity. AM, apprenticeship training programme + match; GR, gradual training; IM = the sum of match intensity; M, match; MR, match + recovery; RC, recovery + control; S, shock load; SM, shock load + match; T, tapering; TL, training load; TLM, training load + match; TLR, training load + recovery; Pre and Post correspond to the samples obtained just at the beginning of the study and at rest, after its completion; McC, Microcycle; Ss, training session or workout; h' Ss/Week, hours of training sessions per week.

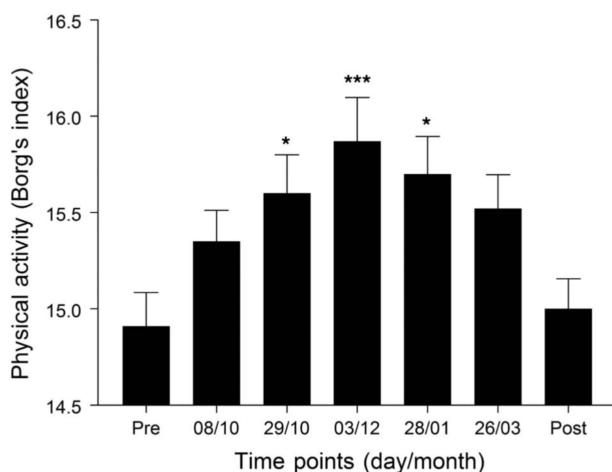

Figure 1. Physical activity evolution of the handball players during the training season in accordance with Borg's scale. Data in the X-axis correspond to the times when the players were analysed along the competition season. Pre and Post correspond to the beginning and the end of the competition season, respectively, and the other values refer to the dates (month/day) during competition (see also Table I). $*P < 0.05$ and $***P < 0.001$ *vs.* Pre and Post.

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fluorometric glutathione and glutathione disulphide measurements (Hissin & Hilf, 1976). Glutathione concentrations were calculated according to standard curves previously prepared. Levels are expressed in μmol · g^{-1} Hb. Haemoglobin (Hb) was measured with the cianometahaemoglobin method (Collier, 1944).

Measurement of glutathione peroxidase and reductase activities

Washed red blood cells were haemolysed in phosphate buffer (10 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid disodium salt, pH 6.25), and centrifuged at 20,000 *g* for 15 min. Supernatants were the used for glutathione peroxidase and reductase measurements in the presence of nicotinamide adenine dinucleotide phosphate (Griffith, 1999). Glutathione peroxidase and glutathione reductase activities are expressed as μmol · min^{-1} · g^{-1} Hb. In both cases, non-enzymatic reduced nicotinamide adenine dinucleotide phosphate oxidation was subtracted from the overall rates(Jaskot, Charlet, Grose, Grady, & Roycroft, 1983).

Plasma cytokine assay

The Milliplex Human Cytokine immunoassay kit (Millipore Corp. Ma, USA) was used to profile expression of three inflammatory mediators (interleukin-1β, interleukin-6 and tumour necrosis factor α and interferon γ). The assay was performed according to the manufacturer's instructions, and the cytokine levels are expressed in ng · l^{-1}.

Nitrite plus nitrate determination

Due to the high instability of the nitric oxide molecule, measurements must be made indirectly by the determination of nitrites, which are the compounds formed after the reaction of nitric oxide with water. Moreover, nitrites are rapidly oxidised to nitrates, which should be reduced again to nitrites with nitrate reductase to obtain a reliable value of the amount of nitric oxide produced during inflammation. The concentration of nitrites was measured following the Griess reaction which coverts nitrite into a coloured azo compound spectrophotometrically detected at 550 nm (Granger & Taintor, 1995). Plasma levels of nitrites plus nitrates are expressed in μmol · l^{-1}.

Quantification of plasma creatine kinase, lactate dehydrogenase, and myoglobin

Serum samples were maintained at 4°C and used for analytical purposes within 12 h after they were collected. Biochemical analyses were done in the Laboratory of Biopathology of the San Cecilio's University Hospital of Granada by routine methods with reagents from Roche using a Cobas 8000 autoanalyser (Roche Diagnostic, Basel, Switzerland). Creatine kinase (CK) was determined by the formation of creatinine in the presence of picric acid; lactate dehydrogenase (LDH) was determined using the International Federation of Clinical Chemistry method by conversion of lactate to pyruvate, and myoglobin by an electrochemiluminescence immunoassay. Serum levels of creatine kinase (U · l^{-1}), lactate dehydrogenase (U · l^{-1}) and myoglobin (μg · l^{-1}), were used as markers for muscle status.

Statistical analysis

Values are expressed as mean ± SE. Analysis of variance (ANOVA) followed by Student's *t* test were used to compare the means between groups (before and after the competition). A *P* value of less than 0.05 was considered statistically significant. All statistical procedures were performed using SPSS version 15.0 (Chicago, Illinois, USA).

Results

The distribution of the training plus competition program is shown in Table I. There is an initial period of progressive, high-intensity training, corresponding to macrocycle I, from microcycles 1 to 16; a second period of physical maintenance, corresponding to the final part of macrocycle I and the first part of macrocycle II, grouping microcycles 17 to 25, and a third period of moderate-high training

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activity, corresponding to microcycles 26 to 36. The different types of training are also depicted in Table I. A shadow area in the bottom of the table reflects the changes in the exercise intensity along the study, and correlates with the data of Figure 1. Exercise intensity, according to Borg's scale (Borg & Dahlstrom, 1962), increased from the beginning of the season, reaching the maximum at the end of macrocycle I ($P < 0.001$); it was maintained during the middle part of the season, and it decreased along macrocycle II. It can be seen that exercise intensity ranged from 14.9 (hard) at the beginning of the study, to 16 (very hard), coinciding with the middle of the competition period, decreasing to 15 (hard) at the end of the study.

Oxidative stress

The profiles of plasma nitrite and lipid peroxidation are shown in Figure 2. Nitrite increased at the 2nd recovery and control microcycle (24.08 ± 1.98 μmol · l^{-1}) compared with values at Pre and 1st recovery and control microcycle (19.7 ± 1.67 μmol · l^{-1} and 17.82 ± 2.08 μmol · l^{-1} respectively, $P < 0.05$), and remained elevated until the 3rd recovery and control microcycle, which reflects an inflammatory response. After that, nitrite levels decreased, reaching the pre-training values at the end of the study (Post), just during the rest period. Plasma lipid peroxidation levels decreased significantly at the 1st recovery and control microcycle compared with the Pre sample (17.4 ± 0.74 μmol · l^{-1} *vs.* 7.26 ± 0.82 μmol · l^{-1}, $P < 0.001$); they remained low during the whole season, recovering the pre-training values at the end of the study (Post), during the rest period

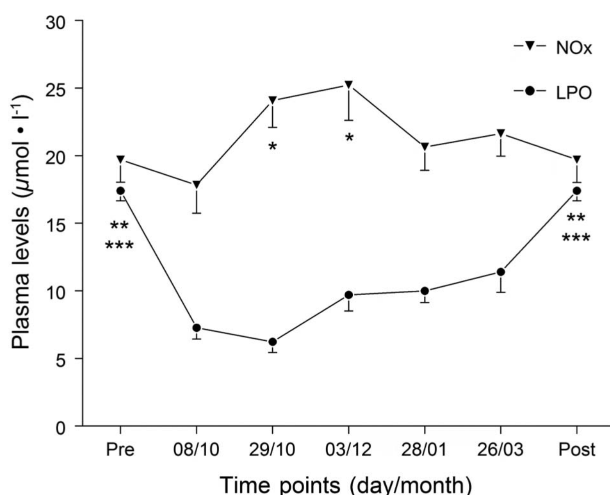

Figure 2. Profile of plasma levels of nitrites (NOx) and lipid peroxidation (LPO) in the handball players during the training season. See legend of Figure 1 for additional information. *$P < 0.05$ *vs.* Pre, 08/10, and Post; **$P < 0.01$ vs. 26/03; ***$P < 0.001$ *vs.* 08/10, 29/10, 03/12, and 28/01.

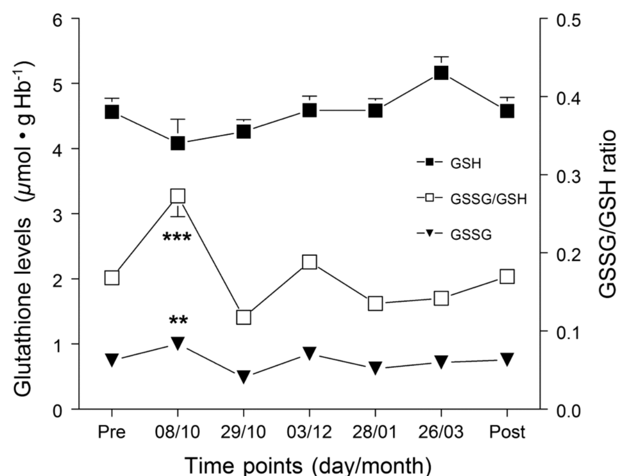

Figure 3. Changes in the erythrocyte levels of glutathione (GSH), glutathione disulphide (GSSG), and in the GSSG/GSH ratio in the handball players during the training season. See legend of Figure 1 for additional information. ***$P < 0.001$ *vs.* Pre, 29/10, 28/01, 26/03, and Post; **$P < 0.01$ vs. Pre, 29/10.

(17.88 ± 1.63 μmol · l^{-1}, $P < 0.001$). The highest nitrite levels and the lowest lipid peroxidation levels took place at the time of highest exercise intensity.

Erythrocyte glutathione disulphide levels increased at the 1st recovery and control microcycle compared with the Pre sample (0.74 ± 0.04 μmol · g Hb^{-1} *vs.* 1.01 ± 0.05 μmol · g Hb^{-1}, $P < 0.01$); it decreased at the 2nd recovery and control microcycle, and remained at the basal levels during the rest of the competition (Figure 3). In contrast, glutathione levels did not change significantly along the season. Consequently, the glutathione disulphide/glutathione ratio increased at the 1st recovery and control microcycle (0.16 ± 0.01 *vs.* 0.27 ± 0.02, $P < 0.001$), suggesting an increased oxidative response, returning to the basal value during the rest of the training season. Exercise also increased slightly the activity of glutathione reductase at the 1st recovery and control microcycle (48.46 ± 3.43 μmol/min · g Hb^{-1} *vs.* 55.61 ± 1.87 μmol/min · g Hb^{-1}, n. s.), but maximal activity of the enzyme was found at the 4th (69.6 ± 2.28 μmol/min · g Hb^{-1}, $P < 0.001$) and 5th (58.59 ± 2.38 μmol/min · g Hb^{-1}, $P < 0.01$) recovery and control microcycles, returning to basal values at the end of the study (Post sample) (Figure 4). The activity of glutathione reductase increased at the 1st recovery and control microcycle (1.83 ± 0.1 μmol/min · g Hb^{-1} *vs.* 2.38 ± 0.14 μmol/min · g Hb^{-1}, $P < 0.05$), returning to its basal activity at the 2nd recovery and control, and remaining low for the rest of the training season.

Except for the initial increase in the glutathione disulphide/glutathione ratio that could be related to the beginning of the training season, other changes in the oxidative markers were unrelated to the physical activity according to the Borg's index.

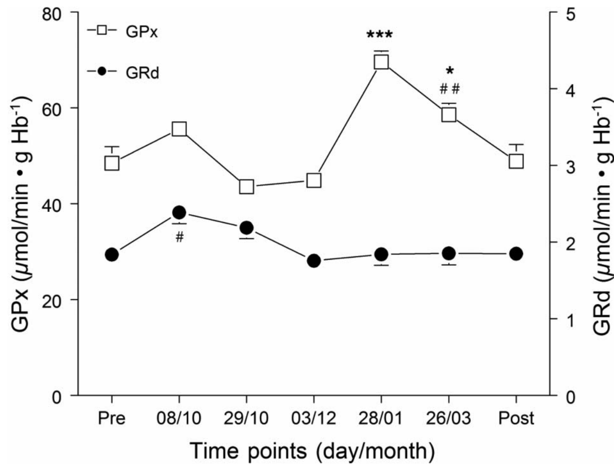

Figure 4. Evolution of glutathione peroxidase (GPx) and reductase (GRd) activities in the erythrocytes of handball players along the training season. See legend of Figure 1 for additional information. *$P < 0.05$ and ***$P < 0.001$ *vs.* Pre, 29/10, 03/12, and Post; ##$P < 0.05$ *vs.* Pre, 03/12, 28/01, 26/03, and Post.

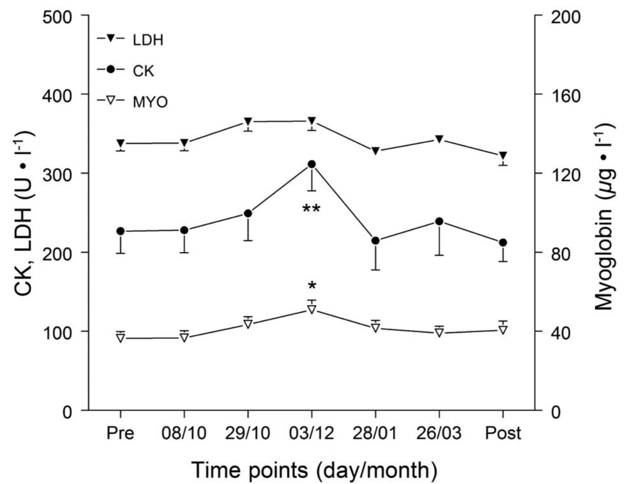

Figure 6. Profile of plasma markers of muscle damage along the training season. LDH and CK are expressed as $U \cdot ml^{-1}$, and myoglobin as $\mu g \cdot l^{-1}$. See legend of Figure 1 for additional information. *$P < 0.05$, and **$P < 0.01$ *vs.* Pre, 08/10, and Post.

Inflammatory status

Plasma pro-inflammatory cytokines IL-6 (1.39 ± 0.15 ng $\cdot$ l^{-1} *vs.* 3.15 ± 0.03 ng $\cdot$ l^{-1}, $P < 0.001$) and IL-1β (1.23 ± 0.17 ng $\cdot$ l^{-1} *vs.* 3.24 ± 0.23 ng $\cdot$ l^{-1}, $P < 0.001$) showed an acute rise at the 1st recovery and control microcycle, with a plateau along the season, then returned to basal values (Figure 5). A similar trend was found for INFγ, with its highest levels found at the 6th recovery and control microcycle (2.26 ± 0.4 ng $\cdot$ l^{-1} *vs.* 12.88 ± 3.67 ng $\cdot$ l^{-1}, $P < 0.05$). Although TNFα tends to decrease with exercise, no significant changes were detected along the study.

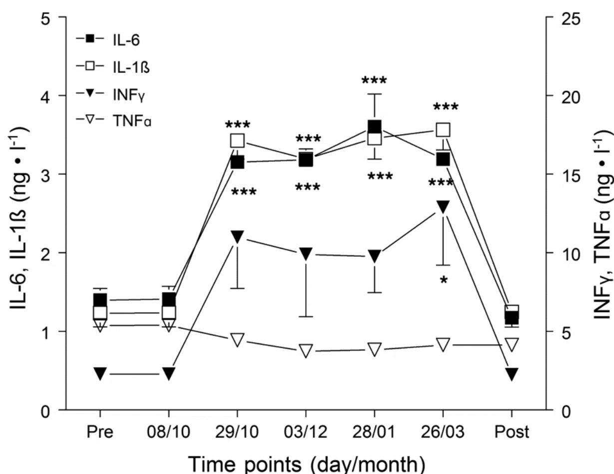

Figure 5. Changes in the plasma levels pro-inflammatory cytokines during the training season. See legend of Figure 1 for additional information. *$P < 0.05$, and ***$P < 0.001$ *vs.* Pre, 08/10, and Post.

Biochemical markers

Levels of LDH (lactate dehydrogenase), CK (creatine kinase), and myoglobin are shown in Figure 6. CK (311.14 ± 33.76 U $\cdot$ l^{-1} *vs.* 226.61 ± 28.12 U $\cdot$ l^{-1}, $P < 0.01$) and myoglobin (50.88 ± 4.83 U $\cdot$ l^{-1} *vs.* 36.35 ± 3.46 U $\cdot$ l^{-1}, $P < 0.05$) levels increased significantly at the middle of the competition season (3rd recovery and control microcycle), compared with their values at Pre and Post samples, coinciding with the maximal exercise intensity. No significant changes were detected for plasma LDH levels.

Discussion

The present results show that well-trained professional handball players react to long-term exercise during the year of competition increasing the inflammatory response, which was supported by the significant increase in pro-inflammatory cytokines and nitric oxide levels. The same players, however, remained well adapted to the redox response because of the low levels of lipid peroxidation and minor changes in the glutathione disulphide/glutathione ratio found along the competition.

The model of training reported here was designed to improve the level of compliance of the players with the competitions during an annual handball championship (Issurin, 2010). The distribution of training plus matches was designed to get the maximal performance at the middle of the season. In fact, during the first period of training and competitions (microcycles from 1 to 16), the exercise intensity of training was becoming greater. So, the players entered the second part of the season, between

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microcycles 15 and 23, with maximal exercise intensity. This period coincides with the transitory peaks in plasma CK and myoglobin. During this time, and until microcycle 25, the players were under training maintenance to optimise their performances, playing a total of 6 matches, somewhat lower than during the first period. At this time, the players maintained their exercise intensity near to the maximal value. Then, the players entered the last competition period, in which the intensity of training was lower, playing 10 matches. Of interest was the observation that, during the last part of the competition season, there was a slight decline in exercise intensity, which might be related to the fact that the players' team had already gained enough points to win the league. The progression in exercise intensity (training plus competitions), correlated well with Borg's scale of exercise intensity (Borg & Dahlstrom, 1962).

There are many ways in which exercise can benefit health (Gissis et al., 2006). Physical exercise augments blood flow and shear stress, improving cardiovascular and endothelial function by upregulation of endothelial nitric oxide synthase (eNOS) activity and increased eNOS-dependent nitric oxide production (Di Francescomarino, Sciartilli, Di Valerio, Di Baldassarre, & Gallina, 2009; Lewis, Dart, Chin-Dusting, & Kingwell, 1999; Paterick & Fletcher, 2001). The elevation of plasma nitrite levels with exercise reported here might, thus, reflect this pathway of nitric oxide production. However, the nitric oxide derived from eNOS during muscle work is locally produced, and it should not influence systemic nitric oxide levels. In this regard, physical activity of high intensity, is related to skeletal muscle damage and subsequent inflammatory response, leading to inducible nitric oxide synthase (iNOS)-dependent nitric oxide production, explaining the high nitrite levels found in our study. The inflammatory response is typical of intense periods of training and competition, and it was also reported in rugby players during a competition season (Finaud et al., 2006).

The existence of an inflammatory response in handball players was also reported elsewhere (Bresciani et al., 2010). These authors reported a transient increase in C-reactive protein during periods of high load. Our data, however, further support a chronic inflammatory status by the high levels of IL-1β and IL-6 and, to a lesser extent, INFγ levels found in the participants of the study. Plasma concentration of IL-6 increases during muscular exercise, followed by the appearance of IL-1 receptor antagonist (IL-1ra) and the anti-inflammatory cytokine IL-10 (Pedersen & Febbraio, 2008). IL-6 is not only an inflammatory cytokine; it exerts significant beneficial effects too. Systemic IL-6 promotes hepatic glucose production and lipolysis in adipose tissue during exercise (Pedersen, 2011), whereas myokine IL-6, the IL-6 produced within the muscle fibres, increases glucose uptake and fat oxidation, and promotes skeletal muscle repair (Philippou et al., 2009). IL-6 can also induce anti-inflammatory responses inhibiting TNFα production (Schindler et al., 1990), a finding that may explain the decreasing TNFα trend in our study. In turn, the changes in INFγ levels reported here may reflect its upregulation in the absence of TNFα (Hodge-Dufour et al., 1998), contributing to the inflammatory response. By contrast, high levels of TNFα promote muscle catabolism and necrosis by a necrosis factor kappa B (NF-kB)-mediated effect (Grounds & Torrisi, 2004), whereas low levels of TNFα stimulate muscle regeneration and myogenesis (Kuru et al., 2003). The fact that TNFα and IL-6 are synthesised in macrophages and in muscle cells (Reid & Li, 2001), may favour the inhibition of the former by IL-6, reducing the skeletal muscle damage with exercise (Pedersen & Febbraio, 2008). Low TNFα levels are also important because they prevent the induction of NF-kB in the presence of elevated IL-1β, which contributes to reduce muscle damage (Grounds & Torrisi, 2004). Changes in IL-6 may depend on the type and duration of exercise, because Teixeira et al. (2009) did not show changes in plasma IL-6 in elite kayakers from pre- to competitive season of one month of duration; it seems that long-term exercise is necessary for the adaptive response to this cytokine.

Regarding the redox status, the levels of plasma lipid peroxidation constitute a sensitive marker for oxidative damage to cell membranes, reflecting plasma hyperoxidative status with exercise (Fatouros et al., 2010; Seifi-Skishahr, Siahkohian, & Nakhostin-Roohi, 2008). Untrained players experienced exacerbated lipid peroxidation levels compared to their trained counterparts, suggesting the existence of adaptive mechanisms to high-intensity exercise in training subjects (Bloomer & Fisher-Wellman, 2008; Seifi-Skishahr et al., 2008). Total antioxidant status and thiobarbituric acid reactive substances increased during short-term acute exercise (Teixeira et al., 2009), but the former did not change during a longer competition season (Finaud et al., 2006). Higher endogenous antioxidant protection in trained athletes compared to sedentary controls might account for lower lipid peroxidation levels found in the former (Fisher-Wellman & Bloomer, 2009; Nakatani et al., 2005). Our results also support this observation, because lipid peroxidation decreased during exercise, increasing at rest (Clarkson & Thompson, 2000). Lipid peroxidation levels reflect the activity of the endogenous peroxide scavengers, including glutathione peroxidase and melatonin, among others. Thus, the initial increase in glutathione peroxidase (and probably the other scavengers which were not measured in this study) could help to reduce lipid peroxidation levels and to

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maintain them at low levels along the study; it may reflect that the endogenous antioxidants react rapidly in training subjects, counteracting reactive oxygen species generation. This observation agrees with our data supporting that well-trained participants were able to maintain their glutathione redox status controlled soon after the initiation of the training season. Measurements of redox changes in glutathione have been routinely performed as an index of the exercise-induced oxidative stress (Douris et al., 2009; Elokda, Shields, & Nielsen, 2005; Fatouros et al., 2010). To maintain the glutathione pool, the activities of the glutathione redox cycle enzymes, glutathione peroxidase and reductase, should be activated during exercise (Fisher-Wellman & Bloomer, 2009). This is exactly what happens in our study. Coinciding with a glutathione disulphide increase at the beginning of the training, there was a slight reduction of glutathione levels; at this time, glutathione reductase was activated to recover the glutathione pool from glutathione disulphide, normalising their levels and maintaining the glutathione disulphide/glutathione ratio under control. A transient glutathione disulphide/glutathione increase during periods of high load was also reported in plasma of handball players (Bresciani et al., 2010). The activity of glutathione peroxidase transiently increased at the end of the season, coinciding with the elevation in the lipid peroxidation levels. This suggests a role for glutathione peroxidase in preventing an excessive oxidative response due to the gradual reduction in the exercise intensity of the players (Inal, Akyuz, Turgut, & Getsfrid, 2001). The glutathione disulphide/glutathione ratio is the best index for the intracellular redox status (Lee & Britz-McKibbin, 2009) and, when it is measured in erythrocytes, reflects the intracellular redox status of the skeletal muscle fibres (Chahbouni et al., 2011). Thus, the changes in the glutathione disulphide/glutathione ratio here reported suggest that the skeletal muscle of well-training participants was able to counteract the oxidative stress induced by exercise.

From these data, one can suggest that well-trained athletes are able to overcome the excess of reactive oxygen species production with their endogenous antioxidant system, thus preventing the oxidative damage to cells and tissues. Interestingly, a study undertaken in elite alpine ski racers reported an absence of changes in endogenous antioxidants during competition, with increased levels of peroxides at the middle of the season, which was associated with their performance (Schippinger et al., 2009). In these conditions, the inflammatory response can be also analysed in terms of its beneficial effects to the skeletal muscle (Jiang & Liao, 2010). In fact, elevated IL-6 plus low TNFα may play a modulatory role of muscle myogenesis and repair during training in well-trained athletes, counteracting or, at least, reducing the deleterious effects of IL-1β and INFγ. Thus, the training model reported here was able to maintain the physical activity of the athletes (Issurin, 2010), whereas the changes in reactive oxygen species and cytokines along the competition may reflect, at least in part, the training status of the participants. Only during the middle of the season, coinciding with the maximal exercise intensity, there was an apparent period of muscle damage, assessed by the temporal CK and myoglobin increase. This observation is in agreement with similar changes in rugby players during competition and rest periods (Finaud et al., 2006). We can conclude that the status of a training individual, in terms of endothelial function, oxidative/nitrosative stress balance, and inflammatory status, depends on the type, intensity, and duration of the physical exercise (Di Francescomarino et al., 2009), and these responses can be modulated through a specific training programme.

Conclusions

The results of this study suggest that well-trained professional handball players are able to adapt to exercise-induced reactive oxygen species production, and thus prevent the hyperoxidative state that normally follows high-intensity exercise. From one competition season to the next, individuals are able to counteract the free radical generation induced by exercise. However, for this to occur, it is probably necessary to apply a training periodisation specifically designed for these athletes, allowing the maintenance of their physical performance along the competition season. Thus, the implementation of the adequate training and periodisation rules reported by Issurin (2010) may benefit athletes significantly. Our training schedule probably prevented the acute reactive oxygen species increases that occur in the skeletal muscle after exercise, responsible for the oxidative damage to the muscle (Fatouros et al., 2010) but not the inflammatory response. Thus, the main question is: why were the athletes unable to control the inflammatory status along the competition? Taking into account some of the beneficial effects of the inflammatory response discussed here, the analysis of the relationships between IL-6/IL-1β/TNFα and muscle repair/damaging effects of exercise (Jiang & Liao, 2010; Philippou et al., 2009), should be further analysed to assess the beneficial versus deleterious consequences of inflammation during exercise.

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