

Increased oxidative stress inflammatory response in juvenile swimmers after a 16-week swimming training

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ABSTRACT

The purpose of this study was to investigate the effects of repetitive swim training on oxidative stress, antioxidant system and inflammatory response in juvenile swimmers. Ten young male swimmers (12.7 ± 0.4 years old) and 19 young female swimmers (12.1 ± 0.3 years old) were included in the study. Swimmers prepared for the races by participating in a sixteen-week training program. Blood samples were obtained at three time points during swim exercise: at the beginning of the training season (before training), after eight weeks of training, and at sixteen weeks of training. The main findings of this study are as follows: (1) Child swimmers experienced greater oxidative stress after exhaustive exercise than untrained subjects as shown by increased NO levels, protein carbonyls, and increased plasma and erythrocyte TBARS levels, as well as decreased protein sulfhydryls in plasma and blood; (2) GSH levels and SOD activity increased significantly, while GSH-Px activity was significantly lower after swimming exercise; and (3) IL-6, IL-8, Hsp-72, and CRP expression levels were elevated in response to repetitive swimming exercise. In conclusion, these findings suggest that swimming training leads to increased oxidative damage and inflammatory response in juvenile swimmers. It remains to be determined how oxidative damage and inflammatory response may be connected under these training conditions in juvenile swimmers.

Keywords: juvenile swimmers; antioxidant status; oxidative stress; inflammatory.

INTRODUCTION

During a resting state, a range of cellular processes, external factors, and/or disease states can lead to the formation of reactive oxygen/nitrogen species (RNOS). In healthy individuals, these RNOS are produced at levels well within the capacity of the body's antioxidant defence system; however, if left unchecked, RNOS can oxidise cell constituents, such as lipids and nucleic acids, leading to deterioration of cellular structural architecture, signalling, and viability [11, 40]. Physical exercise is a stressor known to alter physiologic and metabolic functions responses [36]. The cellular and molecular mechanisms underlying these changes are poorly understood. Although several studies have demonstrated regular physical activity causes concerted adaptations of antioxidant and oxidative damage repair systems [33] several researchers have shown that strenuous exercise can increase the production of RNOS, leading to an oxidative stress condition characterised by lipid peroxidation and protein oxidation products [25]. Strenuous physical exercise has also been implicated in deleterious effects of RNOS on genomic DNA as a result of increased oxygen consumption [8]. The RNOS permeate the cell nucleus and react with DNA [29, 31], which results in DNA strand breaks and the formation of modified nucleotide base and sugar products [9]. The presence of these modified products is a hallmark of oxidative stress, as they are not present during normal nucleotide metabolism. Past studies have primarily utilised the 8-hydroxydeoxyguanosine (8-OHdG) assay and, in some investigations, have shown that elevated 8-OHdG biomarker levels are present in urine and serum samples from individuals engaged in high levels of physical activity [35].

There is scarcity of literature on cellular responses to exercise-induced oxidative damage in childhood. In this study, we first aimed to examine the effect of swimming exercise on oxidative stress in juvenile swimmers. We followed routine biochemical markers of oxidative stress, e.g., lipid peroxidation, MDA, protein carbonyls in plasma and blood, and serum total antioxidant status (TAS). Given the relationship between oxidative stress and inflammatory response, we also wanted to determine if swimming exercise could induce inflammatory response in juvenile swimmers subsequent to oxidative stress. Despite the substantial evidence indicating that strenuous exercise induces oxidative stress and inflammatory response, data correlating antioxidant defence and inflammatory responses in response to exercise are scarce. To shed light on this issue, we studied known inflammatory markers including such as Heat shock protein 72 (Hsp72), Nitric oxide (NO), IL-6, IL-8, and C-reactive protein (CRP) during strenuous, volitional exhaustive exercise in juvenile swimmers.

MATERIALS AND METHODS

This study was carried out in accordance with the Declaration of Helsinki. The study was approved by the ethics committee of Uludag University and Cerrahpaşa Medical Faculty. Ten young male swimmers (12.7 ± 0.4 years old) and nineteen young female swimmers (12.1 ± 0.3 years old) from the reserve team of a club, with an average age of 12.4 ± 0.3 years, volunteered for the study. Female swimmers body mass index 14.8 ± 2.3 while the male swimmers body mass index 15.8 ± 1.6 were found. They were all swimmers on amateur teams. In choosing the volunteers, at least three years of swimming experience, their training program, and the conditions in which they practiced were taken into consideration. Field experiments were performed on the subjects in the 25 m Bursa indoor swimming pool, with an air temperature of 25 ± 2 °C and water temperature of 24 ± 2 °C. Three Casio 2000 chronometers were used for the calculations. Official race rules were applied when determining the swimming times of the subjects. Swimmers prepared for the races by participating in a sixteen-week training program. The swimmers completed the general training period, which included at the beginning of the training season (before training), 6 weeks of aerobic endurance (Indurance-I), 8 weeks of aerobic endurance (Indurance-II), and a 2-week taper (peak) period. Throughout the biochemical experiments, subjects were asked to maintain their usual diets, to get adequate sleep at nights, not to drink coffee or tea or undertake any resistance training before the experiments, and not to use any medication. None of the female subjects had experienced menarche. On the day of the exercise test, the subjects ate a light, carbohydrate-rich breakfast. The exercise test was carried out 2-4 h after breakfast.

Blood sampling and preparation

The study participants were subjected to blood sampling, dietary assessment, anthropometric measurements, and performance measurements at three time points: at the beginning of the training season (baseline), after eight weeks of training (8 w), and at sixteen weeks (16 w) of training. Venous blood samples were collected immediately. At each time point, blood was drawn from the antecubital vein into two evacuated collection tubes-one containing Na₂EDTA (2 ml) and the other heparin (6 ml). From the heparinised tube, one millilitre of blood was immediately pipetted into another tube to measure the extent of DNA damage. Two millilitres of blood from the heparinised tube was evaluated for red cell lipid peroxidation, glutathione, and glutathione peroxidase activity. Remaining blood was centrifuged at 3000 rpm for 10 min to fractionate plasma. Plasma samples were stored at -80 °C for all other biochemical analyses. The EDTA-containing blood was used for haematological analysis. Complete blood cell counts, and haemoglobin (Hb) and haematocrit (Hct) levels were measured in an automated haematology analyser (Sysmex-SE9000, Japan).

Analyses of oxidative stress markers

The 2-thiobarbituric acid reactive substances (TBARS), such as malondialdehyde, which are final products of lipid peroxidation, were detected photometrically. The results of this procedure are given as nmol TBARS/mg protein [10] MDA levels in plasma were measured via the thiobarbituric acid reaction according to the method of Placer et al., (1996). Values of MDA were expressed as $\mu\text{mol/L}$ of plasma. Total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA.

Total protein sulfhydryl (TP-SH) levels were measured spectrophotometrically using Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), with the thiol-disulfide interchange reaction between DTNB and free thiol providing the basis of the spectrophotometric assay [21]. Total protein carbonyl groups were measured as an indication of oxidative damage to proteins. Plasma and erythrocyte protein carbonyl contents were measured by first forming labelled protein hydrazone derivatives using 2,4-dinitrophenylhydrazine (DNPH) [22]. NO_x levels in plasma samples were determined based on the reduction of nitrate to nitrite by vanadium chloride [24]. Nitrite levels (NO₂⁻) levels were measured by the Griess reaction [14]. The absorbance of total nitrite after conversion was measured at 540 nm (Shimadzu UV spectrophotometer, model 1208, Japan).

Assays for antioxidant enzymes

Erythrocyte GSH content was estimated using the method described by Beutler [4]. Erythrocyte SOD activity was measured using a colorimetric method [37]. The separated red blood cells were washed and used to analyse enzyme activities (SOD, GPx, Cat). The haemolysate was mixed with ice-cold 0.05 M phosphate buffer containing 1 mM EDTA. The SOD was extracted from the supernatant using chloroform-ethanol, and measured using a method that exploits the enzyme's ability to inhibit the reduction of nitroblue tetrazolium (NBT) via superoxide generation (by the reaction of photoreduced riboflavin and oxygen). To determine CAT activity, erythrocytes were sonicated in a 50 mM phosphate buffer and the resulting suspension was centrifuged at $3,000 \times g$ for 10 min. The supernatant was used for the enzyme assay. CAT activity was measured by the rate of decrease in H_2O_2 absorbance at 240 nm [1]. Enzyme activity was expressed as u/L. Serum Glutathione peroxidase (GSH-Px) activity was measured indirectly by monitoring the consumption of NADPH at 340 nm [38]. A colorimetric assay was employed to determine plasma total antioxidant status (TAS) (TAS, Randox Laboratories, UK). The results of TAS were expressed as mmol/L. TBARS, carbonyl levels, SOD, CAT, and GSH-Px activity data were expressed in relation to protein concentration estimated using a standard bovine serum albumin curve [23].

Analyses of inflammatory markers

The plasma level of Hsp70 in serum was detected via a commercially available sandwich ELISA (Stressgen Biotechnology, Victoria, BC, Canada). The amount of Hsp 72 in serum was estimated from the calibration curve which ranged from 0.78 to 50 ng/mL. Plasma IL-6 and IL-8 concentrations were determined with an ELISA kit, using 96-well plates (Quantikine High Sensitivity; R&D Systems Minneapolis, MN). The plasma level of CRP was measured immediately with an autoanalyser (Aeroset, Abbott) using a commercial spectrophotometric kit (Scil Diagnostics GmbH, Germany).

Values are reported as the mean \pm SD. All data were normally distributed and underwent equal variance testing. The experiments were analysed with the general linear model of SPSS 11.5 for Windows (SPSS, Chicago, IL). Significance ($p < 0.05$) was determined with a one-way ANOVA, Tukey's HSD test.

RESULTS

All measured oxidative stress biomarker values are shown in Figure 1. As shown in Figure 1, a significant decrease in lipid peroxidation end products (TBARS) in the erythrocytes of juvenile swimmers was observed after eight weeks of swimming exercise training. However, a significant increase in erythrocyte TBARS levels was observed in the juvenile swimmers after sixteen weeks of swimming training. In addition, plasma TBARS levels showed no significant alteration throughout the physical training period. Swimming training also caused increases in NO and total protein carbonyls levels, whereas total protein sulfhydryls decreased significantly throughout the physical training period.

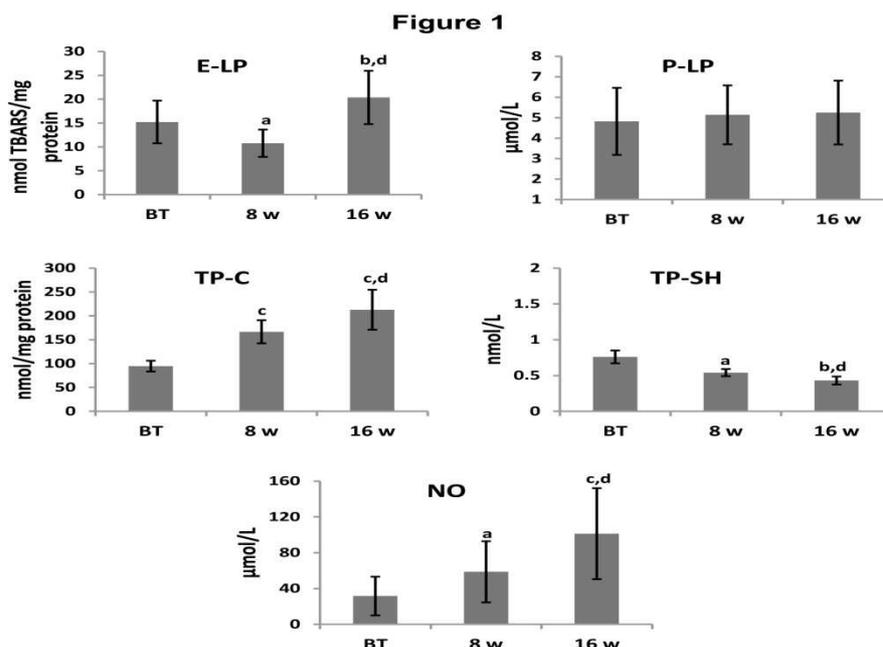


Figure 1: Effect of swim training on oxidative stress markers

Blood samples were obtained before training (BT), at 8 th (8 w) and 16 th weeks (16 w) of swimming exercise. Lipid peroxidation (LP) products as thiobarbituric acid reactive substances (TBARS) were determined in erythrocyte (E-LP) and in plasma (P-LP). Total protein carbonyls (TP-C), total protein sulfhydryl (TP-SH) levels were determined in plasma as described in Methods section. Data are presented as the mean \pm SD. a, $p < 0.05$ with respect to control (BT); b, $p < 0.01$ with respect to control (BT); c, $p < 0.001$ with respect to control (BT); and d, $p < 0.05$ with respect to 8 w values.

Figure 2

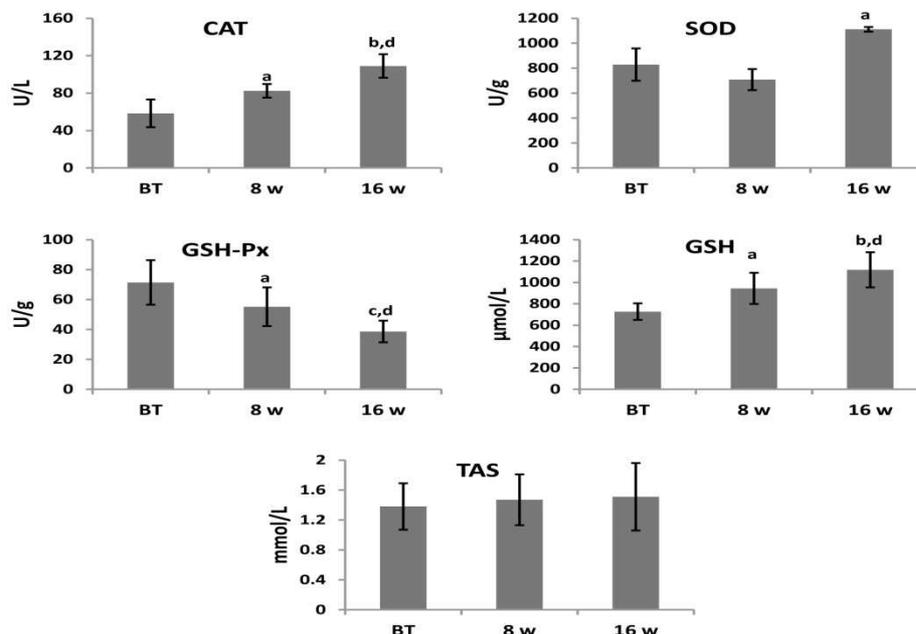


Figure 2: Effect of swim training on antioxidative system.

Blood samples were obtained before training (BT), at 8 th (8 w) and 16 th weeks (16 w) of swimming exercise. Erythrocyte catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities, and glutathione (GSH) levels were determined as described in Methods section. Total antioxidant status (TAS) in plasma was also measured. Data are presented as the mean \pm SD. a, $p < 0.05$ with respect to control (BT); b, $p < 0.01$ with respect to control (BT); c, $p < 0.001$ with respect to control (BT); and d, $p < 0.05$ with respect to 8 w values.

Figure 3

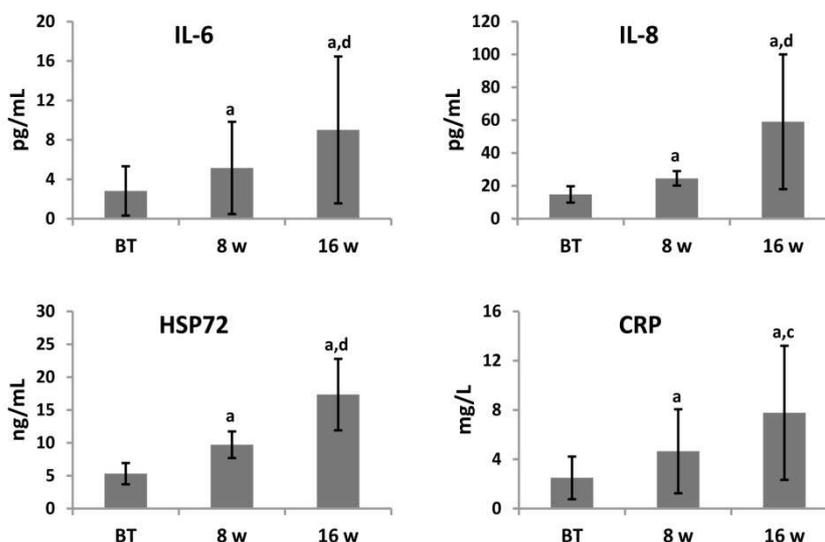


Figure 3: Effect of swim training on inflammatory response markers.

Blood samples were obtained before training (BT), at 8 th (8 w) and 16 th weeks (16 w) of swimming exercise. Heath shock protein 72 (Hsp72), interleukin-6 (IL-6), and interleukin-8 (IL-8) levels were determined in plasma using ELISA. Plasma C-reactive protein (CRP) levels were deremined spectrophotometrically. Data are presented as the mean \pm SD. a, $p < 0.001$ with respect to control (BT); b, $p < 0.01$ with respect to 8 w values; and d, $p < 0.001$ with respect to 8 w values.

DISCUSSION

To examine acute oxidative stress in response to exercise, most researches have assessed various stress markers in blood and urine. We chose to study the oxidant/antioxidant balance in the various components of blood (e.g., red blood cells and plasma) for several reasons. First, blood is a readily available source to study an oxidant/antioxidant imbalance. In addition, it is important to use minimally invasive methods in the case of children, as sampling of muscle tissue is usually not ethically feasible. In blood, it may be most appropriate to evaluate lipid peroxidation levels and carbonylated protein, possibly in erythrocyte membranes. Second, blood is an important pool of antioxidant defences in the body. Third, blood is a readily available medium in order to investigate the putative link between oxidative stress and inflammatory responses [28].

Most authors suggest that competitive athletic activity at an early age may prompt an increase in ROS and a decrease in antioxidants. Previous studies addressing the role of physical exercise in oxidative stress and antioxidant status in juvenile people have shown conflicting results. Two recent reports suggest that acute swimming increases oxidative stress in children, and child swimmers exhibit higher oxidative stress and lower antioxidant capacity compared to untrained counterparts at rest [13]. On the other hand, Cavas and Tarhan [7], found no differences in antioxidant status. Kabasakalis et al. (2009), recently reported that the blood redox status of pubescent swimmers exhibited no significant alterations throughout twenty-three weeks of swimming training. The data presented here demonstrate that the blood markers of oxidative damage to lipids (TBARS) and proteins (carbonyls) were increased as a result of strenuous swimming training in juvenile subjects. We found no significant changes, however, in plasma MDA levels compared to baseline values after the sixteen-week swimming exercise program, which is in disagreement with reports indicating increased plasma MDA after cycling exercise in young people [27]. Our results indicate that ROS production during exercise was not extensive enough to elicit a significant increase in plasma MDA after exercise, because once aldehydes, like MDA, are formed and enter circulation, they are rapidly excreted.

One of the primary objectives of this study was to investigate any alterations in the antioxidant status of blood and plasma. Scavenger enzyme systems and endogenous antioxidants represent a line of defence against oxidative damage to lipids, proteins, and DNA induced by various stimuli, such as exercise [32]. In the present study, exercise caused a significant increase in CAT and SOD activity in erythrocytes. Moreover, erythrocyte GSH-Px activity decreased as a consequence of modified swimming training. We postulate that this improvement in antioxidative protection results from the repetition of a non-damaging mild oxidative stress. In other words, the observed changed in SOD, CAT, and GSH-Px activity may be specifically related to swimming training, since these alterations also occur in both adolescent and young athletes. These data constitutes an interesting opportunity for additional research. Besides activating antioxidant defences, such as SOD and CAT, the presented swimming training regimen does not appear to protect juveniles from the oxidative stress induced by exercise, as indicated by increased levels of TBARS and protein carbonyls. Additionally, we observed increased glutathione (GSH) levels and decreased glutathione peroxidase (GSH-Px) activity in red blood cells (Figure 2). It is our opinion that the increase in blood GSH is probably due to export from peripheral tissues, such as skeletal muscle [19].

Plasma TAS is an accurate index of oxidative stress, which provides a measure of total plasma defences against RNOS [5]. The results of this study revealed a slight, but not statistically significant, increase in TAS levels. This result is in accordance with a study by Kabasakalis et al. [20], in which they reported that a slight increase TAS levels in child swimmers after a 23-week exercise training period. But Gougoura, et al, [13], found lower TAS levels in child swimmers compared to untrained counterparts at rest. Possible explanations for the discrepancy could relate to the methodologies used in the different studies, the time points examined, the levels of training of the participants, or differences in exercise conditions.

Highly reactive NO is produced by a variety of cells and acts as an intracellular messenger in many biological processes. NO has a short biological half-life and is rapidly converted into the stable metabolites nitrite and nitrate. In plasma, nitrite is rapidly oxidised to nitrate. Determination of nitrite and nitrate (NOx) in body fluids like plasma and urine is widely used as a marker of NO production [2]. Some experimental studies have indicated a possible relationship between exercise and NO. There is also evidence that strenuous exercise increases (up to 1.6-fold) the metabolic production of NO in plasma [18]. Thus, we evaluated the NO levels of juvenile swimmers and found training caused an increase in NO levels. This is, to the best of our knowledge, the first clinical evidence suggesting

that NO may be related to increased oxidative stress in juvenile swimmers. NO has been shown to interact with free radicals such as superoxide (O_2^-); it couples with O_2^- to produce peroxynitrite, a compound harmful to cellular structures. Peroxynitrite and other products have been linked to several interactions that may contribute to cellular injury, including lipid peroxidation (e.g., MDA) and protein oxidation (e.g., sulphydrils) [16,17].

Mechanisms for the maturity- and age-dependent changes in inflammatory cytokine responses to exercise are unknown, but may involve oxidative stress. Exercise may also induce inflammatory reactions similar to the acute phase response occurring in injury or infection. Exercise increases cellular metabolism and thus enhances the leakage of oxygen-derived free radicals into plasma [15]. Oxidative stress (i.e., free radicals) is intricately connected to cytokine gene transcription in various tissues. Immunological reactions lead to an augmented generation of reactive oxygen species (ROS) by leukocytes, a mechanism that is partly mediated by cytokines like plasma interleukin-8 (IL-8) and tumour necrosis factor α (TNF α). Exercise-induced changes such as depressed proliferation of lymphocytes, impaired neutrophil function, or DNA damage have been shown to occur in leukocytes themselves and are assumed to be induced in part by oxidative stress [26]. In addition, heat shock protein 72 (Hsp 72) has been implicated as a signalling molecule in the immune response to exercise. A duration- and intensity-dependent role for Hsp72 in the exercise-induced changes of the immune response may be assumed [12]. Studies of the inflammatory cytokine response and exercise in children are scarce. Taking these data into account, we also evaluated the link between swimming exercise and inflammatory response. Plasma levels of Hsp 72 and IL-6 and IL-8 were significantly higher in our juvenile swimmers compared to resting situations. Recent symposium reports revealed that IL-6 and IL-8 can be produced in skeletal muscle during exercise in children [34]. Walsh and colleague demonstrated that acute exercise can increase Hsp72 in the peripheral circulation [39]. They hypothesized that an acute bout of physical exercise would increase Hsp72 gene or protein expression in contracting skeletal muscle, which would then be released, resulting in an increase in Hsp72 within the blood. In addition, recent evidence suggests that Hsp72 can stimulate cytokine production in immune cells as demonstrated by the increased pro inflammatory cytokines including IL-1 β , TNF- α , and IL-6 [3]. Moreover, superoxide anions can lead to inflammatory cytokine production. They have been shown to interact with GSH-Px, reducing its activity. Furthermore, reaction with NO can lead to the formation of peroxynitrite and hydroxyl radicals, which would amplify the inflammatory process. Moreover, increased generation of superoxide radical has been shown to correlate with DNA damage [6].

CONCLUSION

In conclusion, these findings suggest that swimming training leads to increased oxidative damage and inflammatory response in juvenile swimmers. It remains to be determined how oxidative damage and inflammatory response may be connected under these training conditions in juvenile swimmers.

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