Reverse Effects of DPI Administration Combined With Glutamine Supplementation on Function of Rat Neutrophils Induced by Overtraining

Jingmei Dong, Peijie Chen, Qing Liu, Ru Wang, Weihua Xiao, and Yajun Zhang

**Purpose:** To examine the excessive reactive oxygen species (ROS) mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the combined effect of glutamine supplementation and diphenyleneiodonium (DPI) on the function of neutrophils induced by overtraining. **Methods:** Fifty male Wistar rats were randomly divided into 5 groups: control group (C), overtraining group (E), DPI-administration group (D), glutamine-supplementation group (G), and combined DPI and glutamine group (DG). Blood was sampled from the orbital vein after rats were trained on treadmill for 11 wk. Cytokine and lipid peroxidation in blood plasma were measured by enzyme-linked immunosorbent assay. The colocalization between gp91phox and p47phox of the NADPH oxidase was detected using immunocytochemistry and confocal microscopy. The activity of NADPH oxidase was assessed by chemiluminescence. Results: NADPH oxidase was activated by overtraining. Cytokine and lipid peroxidation in blood plasma and the activity of NADPH oxidase were markedly increased in Group E compared with Group C. Neutrophil function was lower in Group E than Group C. Both lower neutrophils function and higher ROS production were reversed in Group DG. The glutamine and DPI interference alone in Group D and Group G was less effective than DPI and glutamine combined in group DG. Conclusion: Activation of NADPH oxidase is responsible for the production of superoxide anions, which leads to excessive ROS and is related to the decrease in neutrophil function induced by overtraining. The combined DPI administration and glutamine supplementation reversed the decreased neutrophil function after overtraining.

**Keywords:** NADPH oxidase, diphenyleneiodonium, respiration burst, phagocytosis function, radical oxygen species, ROS

Overtraining is considered a state of fatigue in which athletes fail to improve or, even worse, deteriorate their performance despite continuous training. It is due to physical and mental overloading that disturb the physiological and psychological states of the athletes who fail to exert their best performance (Amit, Ishita, & Papadopoulou, 2012; Nieman, 2012). Various syndromes have been proposed to identify overtraining, called overtraining syndrome or overreaching, which has received much attention from researchers over the last 2 decades (Lancaster & Halson, 2004). Overtraining syndrome is a complex state that involves a large variety of signs and symptoms. One sign of overtraining syndrome is suppressed immune function, with an increased incidence of upper respiratory tract infection (Smith, 2003), increased humeral immunity, and suppressed cell-mediated immunity (Lakier Smith, 2003). Smith (2004) also suggests that excessive training causes repetitive tissue trauma, to muscle and/or connective tissue and/or bony structures, which is caused by a chronic systemic inflammatory process mediated by proinflammatory cytokines produced mainly by neutrophils and macrophages. Neutrophils play important roles in both the antibacterial host-defense mechanism and the pathogenesis of tissue injury and inflammatory responses. Neutrophils rely on a variety of radical oxygen species (ROS; e.g., O$_2^-$, H$_2$O$_2$, OH) mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to damage the bacterial constituent. Although ROS generated by the neutrophil respiratory burst is vital in clearing away muscle tissue that has been damaged by exercise, it is also responsible for causing further damage (Tiidus, 1998). A review of studies by Peake and Suzuki (2004) also indicated that the generation of large quantities of toxic ROS by hyperactivation of NADPH oxidase in neutrophils could lead to excess oxidative stress during intense exercise, which attenuates the function of neutrophils and contributes to inflammation. Therefore, it is necessary to give careful consideration to neutrophil functions, which are associated with ROS mediated by NADPH oxidase induced by overtraining.
Glutamine is the most abundant free amino acid in human muscle and plasma, which is used at very high rates by leukocytes to provide energy and optimal conditions for nucleotide biosynthesis (Agostini & Biolo, 2010). Glutamine has been proven to delay the occurrence of spontaneous apoptosis in neutrophils (Pithon-Curi et al., 2003). Overtraining is frequently associated with reduced availability of glutamine and decreased immunocompetence. After exercise, reduced glutamine availability is considered a marker of overtraining. Parry-Billings et al. (1992) also indicate that overtraining syndrome is associated with a chronic reduction in plasma glutamine levels. Therefore, many studies have focused on the effect of glutamine supplementation and glutamine metabolism on the neutrophil function induced by exercise. Some studies showed that when exercise was prolonged, both plasma glutamine and neutrophil function were decreased 1 and 2.5 hr postexercise (Robson, Blannin, Walsh, Castell, & Gleeson, 1999). Some studies demonstrated that glutamine supplementation caused a significant increase in neutrophil phagocytic capacity in both resting and acutely exercised rats (Lagranha et al., 2005; Quindry, Stone, King, & Broeder, 2003). Therefore, it seems that glutamine supplementation repairs exercise-induced damage in neutrophil function. But these studies may ignore the fact that exercise increases the production of ROS and glutamine administration causes a further increase in the production of reactive oxygen metabolites and nitric oxide in neutrophils (Lagranha et al., 2005). Our previous study proved that excessive ROS in neutrophils can lead to DNA damage induced by overtraining (Dong et al., 2011). Therefore, we have hypothesized that the effect of a single glutamine supplementation on neutrophil function remains controversial and varies with the duration and intensity of exercise or the state of oxidative stress in athletes. It may help in moderate-intensity exercise but may not for overtraining.

Diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, shows potent anti-inflammatory and neuroprotective effects by inhibiting microglial activation to decreased ROS production, the subsequent release of proinflammatory cytokine TNF-α, and the production of nitric oxide (Qian et al., 2007). DPI is also considered an antioxidant playing a pivotal role in the pathology of arthritis, which can be therapeutically targeted by NADPH oxidase inhibitors (Miesel, Sanocka, Kurpisz, & Kröger, 1995). We designed an overtraining protocol in which Wistar rats were treated with DPI and glutamine simultaneously. On one hand, NADPH oxidase has been a target for regulating the production of endogenous ROS in neutrophils (El-Benna, Dang, & Gougerot-Pocidalo, 2008). On the other hand, glutamine utilization is critical in providing energy and biosynthetic precursors for the proliferation of neutrophils (Moinard, Caldefie-Chezet, Walrand, Vasson, & Cynober, 2002). After 11 weeks of increased-load treadmill training, we measured the plasma cytokine levels related to neutrophil function, respiratory burst, and phagocytosis in vitro and the colocalization of gp91phox and p47phox, which is the major subunit of NADPH oxidase, to examine neutrophil activation and observe the single and interactive reaction of both the NADPH oxidase inhibitor and glutamine supplementation on neutrophil function and activation of NADPH oxidase induced by overtraining.

### Methods

#### Series 1: Animals and Overtraining Protocol

Twenty-four male Wistar rats (mean weight, 230 ± 20 g; SIPPF BK Co., Ltd., Shanghai, China) were used in the study. A motorized treadmill (DSPT202, Qianjiang Technology Co., Hangzhou, China) with five individual lanes without incline was used in a dark room with dim light. After a week of adaptive feeding and a week of walking on a treadmill (5–10 m/min), we selected the animals that voluntarily ran (n = 20) from all animals to permit 5 animals to run at the same time, which were randomly divided into the control group (C) and overtraining group (E). Group C was kept rested and sampled under conditions similar to those of the exercised rats. Group E was trained by the model of overtraining that is referred to as Hohl training (Hohl et al., 2009) and our previous study (Dong et al., 2011). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Shanghai University of Sports. The significant lowering of hemoglobin, cortisone, and testosterone in Group E was regarded as a success for the overtraining protocol because exercise training is associated with hormonal-neuronal regulation, which may have affected the hypothalamus axis and be related to cytokines (Steinacker, Lormes, Reissnecker, & Liu, 2004). A review by de Graaf-Roelfsema, Keizer, Breda, Wijnberg, and Kolk (2007) reported that repeated bouts of exercise are suggested to provide a way of detecting subtle changes in hormonal responses in individual athletes, which may make them an important tool in detecting early overtraining.

#### Series 2: Evidence of Activation of NADPH Oxidase in Neutrophils Induced by Overtraining

Based on the Series 1 overtraining protocol, we wanted to determine whether neutrophil respiratory burst, mediated by NADPH oxidase, could be activated by overtraining. After the final training session, rats were decapitated and peripheral blood (1 ml, heparin anticoagulation) was immediately collected. We separated the neutrophils from the blood to examine the colocalization of gp91phox and p47phox of NADPH oxidase by immunocytochemistry and laser confocal microscopy.

#### Series 3: DPI Administration Combined With Glutamine Supplementation

Based on our findings from Series 1 and Series 2, the combined effect of DPI and glutamine on the function...
of neutrophils induced by overtraining was examined. Another 50 male Wistar rats were randomly divided into five groups: control group (C), overtraining group (E), overtraining and DPI-administration group (D), overtraining and glutamine-supplementation group (G), and overtraining with combined DPI-administration and glutamine-supplementation group (DG). Thirty minutes before training, the rats in Group D were intraperitoneally injected with DPI (0.2 mg/kg, dissolved in 5% glucose, Sigma-Aldrich, USA; Siragy & Huang, 2008). Rats in Group G were intragastrically administered glutamine (dissolved in 0.9% NaCl, Sigma-Aldrich, USA; Cruzat & Tirapegui, 2009), and rats in group DG were given both DPI and glutamine from the fifth week. The doses and concentrations of DPI injected and glutamine supplemented during the deferent stage of exercise are shown in Table 1. The placebo (5% glucose solution and 0.9% NaCl) was given to Groups C and E simultaneously.

Body Weight and Blood Collection

The dynamic changes in weight of the rats were monitored during the 11 weeks of treadmill exercise. Blood samples were collected from the fossa orbitalis venous plexus. The animals were sacrificed 36–40 hr after the last session of exercise to avoid the acute effect of exercise. Whole blood (3 ml) was collected from the ruptured orbital sinus into a capillary tube (containing heparin). From this blood, 200 μl were used directly to assay the respiration burst and phagocytosis function in vitro. Eight hundred microliters were immediately centrifuged at 450 g for 10 min at 4 °C to separate plasma and were stored at –70 °C for measuring cytokines. Two milliliters were used for the preparation of neutrophils.

Preparation of Peripheral Blood Neutrophil Granulocytes

Two milliliters of whole blood were collected for the preparation of neutrophil granulocytes, which were carefully layered over 2 ml of the density gradient Polymorphprep (Axis-Shield, Oslo, Norway). According to the manufacturer’s instructions, samples were centrifuged for 30 min at 500 g. Two leukocyte bands were visible. The top band contained the fraction of mononuclear cells, and the lower band contained the fraction of polymorphonuclear leukocytes (PMNs). The layer of PMNs was collected by pipetting and washed with 0.45% NaCl, followed by the addition of the same volume of 0.9% NaCl to restore osmolarity. The viability of cells (99%) was assessed by trypan blue staining (0.4% trypan blue in PBS, Sigma-Aldrich, USA). PMN preparations contained 95% neutrophils, assessed by hematoxylin/eosin staining on smears, whereas only a few basophil and eosinophil granulocytes could be detected. No monocytes or lymphocytes contaminated the preparation. The final concentration of 1 × 10^7 cells/ml was determined by a cell-counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-königshofen, Germany). Finally, cells were resuspended in RPMI 1640 (1 ml) culture medium (Nissui Pharmaceutical, Tokyo, Japan) with 20% fetal calf serum, and the index in Group D was used for the measurement of intracellular ROS and NADPH oxidase activity.

Immunofluorescence Staining and Confocal Microscopy

In Series 2, 1 ml of blood was collected immediately after the last exercise and PMNs isolated. Cell samples were prepared as Liu et al. (2008) previously described. Briefly, PMNs were cultured onto four-chamber slides precoated with 1% gelatin until nearly confluent and fixed with 1% formaldehyde in phosphate-buffered solution (PBS) at room temperature for 30 min. Cells were washed three times with PBS and permeabilized in 0.1% TritonX-100 in PBS for 10 min. Slides were blocked with 20% fetal calf serum in PBS for 30 min at room temperature. Cells were washed with 0.1% bovine serum albumin/PBS three times with gentle shaking and were incubated at 37 °C for 2 hr with anti-p91phox rabbit polyclonal antibody (H-60, Santa Cruz Biotechnology) and anti-p47phox goat polyclonal antibody (C-20, Santa Cruz Biotechnology) at a 1:500 dilution in PBS containing 10% bovine serum albumin and 0.1% Tween-20. Cells were stained with fluorescein-isothiocyanate-conjugated rabbit anti-goat IgG (81-1611, Zymed) or Cy5-conjugated goat antirabbit IgG (81-6116, Zymed) at a ratio of 1:100 in PBS containing 10% bovine serum albumin and 0.1% Tween-20 at 37 °C for 1 hr. After extensive washing in PBS, the cells were mounted on slides using a diamidinophenyl-indole mounting medium. The stained cells were observed under a confocal laser-scanning microscope (LSM 510 META, Zeiss).

Table 1  Doses of Diphenyleneiodonium (DPI) Injected and Glutamine Supplementation During Exercise

<table>
<thead>
<tr>
<th>Weeks</th>
<th>DPI</th>
<th>Glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–8</td>
<td>0.1 mg/kg once a day</td>
<td>0.8 g · kg⁻¹ · day⁻¹ intragastric administration</td>
</tr>
<tr>
<td>9</td>
<td>0.2 mg/kg once a day</td>
<td>0.9 g · kg⁻¹ · day⁻¹ dissolved in drinking water</td>
</tr>
<tr>
<td>10</td>
<td>0.2 mg/kg twice a day</td>
<td>1 g · kg⁻¹ · day⁻¹ dissolved in drinking water</td>
</tr>
<tr>
<td>11</td>
<td>0.3 mg/kg twice a day</td>
<td>1.1 g · kg⁻¹ · day⁻¹ dissolved in drinking water</td>
</tr>
</tbody>
</table>
Determination of Intracellular ROS With Flow Cytometry

Intracellular ROS was determined by a DCFH-DA assay as Bass et al. (1983) described, with minor modifications. The fluorogenic probe 5-(and 6-)-chloromethyl-2′,7′-dichlorodihydrofluoresceindiacetate acetyl ester (CM-H2DCFDA) was used as the molecular probe.

We evaluated intracellular ROS with an ROS assay kit (Genmed Scientific Inc., USA) according to the manufacturer’s recommendations. First-flow-length fluorescence and square were monitored by flow cytometry (Beckman Coulter, USA) of 10,000 cells of each sample within a gated area previously determined to be positive cells. The amount of intracellular ROS generation, paralleled by an increase in fluorescence intensity, was calculated as a percentage of control cells.

Measurements of NADPH Oxidase Activity

We measured the activity of NADPH oxidase in PMNs by monitoring the chemiluminescence (Hitachi Medical Corp., Sirius-2, Funakoshi, Tokyo, Japan) using a lucigenin assay kit (Genmed Scientifics Inc., USA, GMS50095.1V.A). According to the manufacturer’s protocol, NADPH oxidase activity was expressed as relative light units per minute.

Respiratory Burst and Phagocytosis Assay of Blood PMNs

Neutrophil oxidative burst activity and phagocytosis function were measured with a commercially available burst test kit and Phago test kit (Glycotope Biotechnology, GmbH, Germany) using flow cytometry according to the manufacturer’s protocol (Huttemann, Jung, van Hout, & Sakka, 2006). Whole blood (100 μl) was used for the analysis. Briefly, blood samples were incubated at 37 °C with phorbol myristate acetate as the stimulus. After 10 min, the reaction was stopped; leukocytes were then isolated by lysis of erythrocytes and fixed. Formation of the green fluorescent rhodamine (the oxidant product of 123-DHR) was assessed by a fluorescence-activated cell scan-flow cytometer (Beckman Coulter) with a filter wavelength of 488 nm. For each sample, 60,000 cells were collected; granulocytes were identified by their forward and side light-scatter characteristics. The PMN respiratory burst activity of each sample was expressed as a percentage of oxidation cells. Furthermore, mean fluorescence intensity as a measure of metabolic activity was determined (see Figure 1).

Meanwhile, whole blood (100 μl) was incubated with fluorescein-isothiocyanate-labeled E. coli bacteria at 37 °C. Phagocytosis was stopped by placing the samples on ice and adding quenching regents, which enables the discrimination between attachment and internalization of bacteria by quenching the fluorescein isothiocyanate fluorescence of surface-bound bacteria, leaving the fluorescence of internalized particles unaltered. After two washing steps with washing reagents, erythrocytes were removed by the addition of lysis solution. Before analysis, DNA-staining solution was added. Sixty thousand leukocytes were collected by flow cytometry (Beckman Coulter) using the blue-green excitation light (488 nm).

Figure 1 — Change in rats’ weight during 11 weeks of treadmill exercise.
The percentage of cells that had performed phagocytosis, in addition to their mean fluorescence intensity, was analyzed by counting the number of events.

Assay of the Concentration of Cytokines in Rat Blood Plasma

The concentrations of myeloperoxidase (MPO), malondialdehyde (MDA), granulocyte-colony-stimulating factor (G-CSF), interleukin-1β (IL-1β), interleukin-6 (IL-6), cytokine-induced neutrophil chemoattractant (CINC), and tumor necrosis factor-α (TNF-α) in plasma were determined by commercial kits according to the manufacturers’ instructions. The interassay coefficient of variation was kept less than 10%. The blank well was taken as zero, and absorbance was read at a 450-nm wavelength using a microplate reader (Bio-Rad 550, Bio-Rad Laboratories, Hercules, CA). The corresponding sample concentration was calculated according to the standard curve.

Statistical Analysis

Statistical tests were performed with SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL). All data are expressed as M ± SD. Differences among groups were analyzed by ANOVA with a post hoc Bonferroni/Dunn multiple-comparisons test. A value of p < .05 was considered statistically significant. Pearson’s correlation and stepwise regression analysis were used to measure the correlation between activity of NADPH oxidase, MDA and MPO levels in plasma, respiratory burst, phagocytosis function, and production of ROS in different groups.

Results

Change in Weight During 11 Weeks of Treadmill Exercise

As shown in Figure 1, in the 11 weeks of treadmill exercise, we measured the rats’ weight every weekend and found that the weight of the rats in Group C slowly increased during the exercise. The weight of the rats in group E increased during 7 weeks and maintained from the 7th week to the 11th week, but compared with Group C, it declined dramatically after 11 weeks.

Colocalization Between gp91phox and p47phox of the NADPH Oxidase Subunit in Group E Using Immunocytochemistry and Confocal Microscopy Induced by Overtraining

In Series 2, to find evidence for activation of PMNs, we used immunofluorescence staining to determine whether overtraining induces activation of NADPH oxidase. Translocation of the cytosolic subunit p47phox to the membrane is a key step in NADPH oxidase activation (Johnson et al., 1998). We used antigp91phox (red fluorescence) combined with an antip47phox (green fluorescence) antibody to detect the translocation of the p47phox subunit. As shown in Figure 2(A) and 2(B), p47phox was distributed evenly between the cytoplasm and the nucleus of cells in Groups C and D, and no distinct accumulation was detected on the cell membrane, indicating that the majority of p47phox was localized in the cytoplasm of PMNs, but in Figure 2(C), the p47phox subunit was translocated to the cell membrane and colocalized with gp91phox (arrow indicating cells in merged figures, yellow), suggesting that NADPH oxidase was activated in PMNs induced by overtraining.

Concentration Levels of G-CSF, CINC, IL-1β, IL-6, and TNF-α in Rat Plasma After Overtraining

As shown in Table 2, the concentration of cytokines in rat plasma increased 36 hr after overtraining, compared with Group C (p < .01), except for TNF-α (p < .05). The concentration of IL-1β after overtraining increased in all groups (Group D, p < .01; Group E, p < .05; Group DG, p < .01). The level of CINC of plasma in Groups G and DG increased (p < .05). The rest index of plasma in Groups D, G, and DG did not change after overtraining.

Figure 2 — (A and B) No colocalization between gp91phox and p47phox of the NADPH oxidase subunit in the control Group C shown by immunocytochemistry and confocal microscopy (x200). (C) Colocalization between gp91phox and p47phox of the NADPH oxidase subunit in Group E using immunocytochemistry and confocal microscopy (x200). Note that the journal prints in black and white only. This figure is reproduced in color in the online version of the article: http://journals.humankinetics.com/IJSNEM
Concentration of MDA and MPO in Plasma After Overtraining

The concentration of MDA in plasma is shown in Figure 3(A). Compared with Group C, the level of MDA in Group E ($p < .01$) and Group G ($p < .05$) markedly increased but did not significantly change in Group D and Group DG. Compared with Group E, the level of MDA in Groups D ($p < .05$) and DG ($p < .01$) decreased and the level in Group G did not change. Figure 3(B) shows the concentration of MPO in different groups after overtraining. Compared with Group C, the concentration of MPO in plasma in Groups E ($p < .01$) and G ($p < .01$) markedly increased, while that of the other groups did not change. Furthermore, the concentration of those in Groups D ($p < .01$), G ($p < .05$), and DG ($p < .05$) decreased compared with Group E.

Activity of NADPH Oxidase in Neutrophils After Overtraining

The activity of NADPH oxidase was measured by chemiluminescence. The results are shown in Figure 4(A). The activity of NADPH oxidase in Groups E and G increased significantly ($p < .01$) after overtraining. Compared with Group E, the activity of NADPH oxidase in Group D ($p < .01$) and in Group DG ($p < .05$) decreased compared with Group E.

Production of Intracellular ROS of PMNs Induced by Overtraining

The intracellular ROS were detected by flow cytometry. The fluorescence intensities of 10,000 cells were analyzed, as shown in Figure 4(B). Compared with the rest Group C, after overtraining, the generation of ROS in Groups E and G significantly increased ($p < .01$), while that of Group DG increased significantly ($p < .05$) and that of Group D experienced no change. In addition, ROS generation in Groups D ($p < .01$) and DG ($p < .05$) decreased compared with that of Group E.

Change of Respiratory Burst and Phagocytosis Function of PMNs Induced by Overtraining

A flow-cytometric phagocytosis test and burst test were used to evaluate the phagocytic activity and respiratory burst, respectively, of blood PMNs after overtraining. Phorbol myristate acetate was used to stimulate the oxidation of PMNs. The percentage of oxidizing cells that respond to phorbol myristate acetate and mean fluorescence intensity is shown in Figure 5(A). Phagocytic activity was expressed as mean fluorescence intensity and the phagocytic index, which was calculated as the percentage of the number of fluorescent cells (integrated from the first minimum to the end of the fluorescence distribution) to the total number of neutrophils counted. After overtraining, the percentage of oxidative cells, as well as the phagocytic index and mean fluorescence intensity, in Group E decreased ($p < .05$), as shown in Figure 5(B). There was a decreasing trend in Groups D and G, and there was an increasing trend in Group D, but they were not statistically significant compared with Group C. There was an increase in Groups D, G, and DG ($p < .05$) compared with Group E.

Discussion

Successful Construction of Overtraining Protocols

In this study, we used Hohl et al.’s (2009) overtraining protocol. According to Cunha, Ribeiro, and Oliveira’s (2006) review, overtraining included lesion and muscle weakness, cytokine activation, hormonal and hematological alterations, mood swings, psychological depression, and nutritional problems that may lead to loss of appetite and cause diarrhea. As shown in Table 3, significantly lower hemoglobin, cortisone, and testosterone in the control exercise groups were considered indications of

Table 2 Change of Cytokines in Rat Plasma After Overtraining ($M \pm SD, n = 8$ per Group)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Group C</th>
<th>Group E</th>
<th>Group D</th>
<th>Group G</th>
<th>Group DG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β, pg/ml</td>
<td>196.09 ± 41.66</td>
<td>385.33 ± 38.80**</td>
<td>77.79 ± 9.05**</td>
<td>246.89 ± 5.31*</td>
<td>268.40 ± 37.04**</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>422.89 ± 35.91</td>
<td>517.53 ± 35.64**</td>
<td>436.50 ± 28.36</td>
<td>445.95 ± 43.20</td>
<td>429.56 ± 29.06</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>462.06 ± 74.74</td>
<td>569.17 ± 83.73*</td>
<td>530.87 ± 28.68</td>
<td>493.79 ± 47.09</td>
<td>469.58 ± 32.24</td>
</tr>
<tr>
<td>CINC, ng/L</td>
<td>154.28 ± 28.51</td>
<td>378.39 ± 96.53**</td>
<td>131.91 ± 12.97</td>
<td>224.67 ± 4.73*</td>
<td>223.24 ± 19.09*</td>
</tr>
<tr>
<td>G-CSF, ng/L</td>
<td>179.72 ± 30.42</td>
<td>347.01 ± 89.97**</td>
<td>266.24 ± 93.92</td>
<td>225.37 ± 54.93</td>
<td>243.65 ± 17.89</td>
</tr>
</tbody>
</table>

Note. IL = interleukin; TNF = tumor necrosis factor; CINC = cytokine-induced neutrophil chemoattractant; G-CSF = granulocyte-colony-stimulating factor.

* $p < .05$, ** $p < .01$ compared with Group C.
Figure 3 — The concentration of (A) malondialdehyde (MDA) and (B) myeloperoxidase (MPO) in rat plasma after overtraining in all groups: control group (C), overtraining group (E), overtraining and diphenyleneiodonium (DPI)-administration group (D), overtraining and glutamine-supplementation group (G), and overtraining combined with DPI-administration and glutamine-supplementation group (DG). *p < .05, **p < .01 compared with Group C. ▼p < .05, ▼▼p < .01 compared with Group E. M ± SD, n = 8 per group.
Figure 4 — Comparison of (A) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and (B) reactive oxygen species (ROS) generation in different groups induced by overtraining: control group (C), overtraining group (E), overtraining and diphenyleneiodonium (DPI)-administration group (D), overtraining and glutamine-supplementation group (G), and overtraining combined with DPI-administration and glutamine-supplementation group (DG). *p < .05, **p < .01 compared with Group C. ▼p < .05, ▼▼p < .01 compared with Group E. M ± SD, n = 8 per group.
Figure 5 — (A) Respiratory burst and (B) phagocytosis function of polymorphonuclear leukocytes after overtraining in vitro (median percentage of oxidizing cells, range, and \( M \pm SD \)) in different groups: control group (C), overtraining group (E), overtraining and diphenyleneiodonium (DPI)-administration group (D), overtraining and glutamine-supplementation group (G), and overtraining combined with DPI-administration and glutamine-supplementation group (DG). *\( p < .05 \), **\( p < .01 \) compared with Group C. ▼\( p < .05 \), ▼▼\( p < .01 \) compared with Group E. \( M \pm SD, n = 8 \) per group.
success of the overtraining protocol in our experiment (Dong et al., 2011). In addition, we observed the reaction ability and behavioral response of rats to be sharply slowed down, and the color pattern was dark. Meanwhile, weak muscle contraction after stimulation and a sudden decrease in motor capacity were used as the criteria for exhaustion. This training protocol had a key feature. The progressive load increase was accomplished by increasing the frequency of training rather than the treadmill speed. The method decreased the risk of injury and promoted recovery. These features may be beneficial for building a successful overtraining protocol.

**Cytokine Secretion Response to Neutrophils Induced by Overtraining**

In immune systems, neutrophils produce superoxide anion O$_2^-$, which generates other ROS such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), and hypochlorous acid (HOCl), together with microbicidal peptides and proteases. These processes are called the respiratory burst of PMNs mediated by NADPH oxidase, which is composed of two transmembrane proteins (p22$^{\text{phox}}$ and gp91$^{\text{phox}}$/NOX$_2$, which form the cytochrome b$_{558}$), three cytosolic proteins (p47$^{\text{phox}}$, p67$^{\text{phox}}$, p40$^{\text{phox}}$), and a GTPase (Rac1 or Rac2). El-Benna, Dang, and Gougerot-Pocidalo (2008) found that NADPH oxidase activation in phagocytes can be induced by a large number of soluble and particulate factors such as neutrophil adhesion, proinflammatory cytokines, lipopolysaccharide, and other agents. Exercise, as a kind of excitation, could induce neutrophil activation (Peake & Suzuki, 2004). We detected the removal of p47$^{\text{phox}}$ from the cytoplasm to the membrane and colocalization with the gp91$^{\text{phox}}$ in NADPH oxidase using immunocytochemistry and confocal microscopy immediately after overtraining, which is evidence of activation of neutrophils induced by overtraining. This finding indicates that overtraining activates the neutrophils and induces the consequent immune response.

TNF-$\alpha$, an important inflammatory mediator produced by activated monocytes and macrophages, plays a role in many aspects of innate immunity (Kilpatrick, Sun, Li, Vary, & Korchak, 2010). We selected the CINC rather than IL-8 in our studies because rodents lack a homolog of IL-8 that shares similar properties with human IL-8 (Ramos, Heluy-Neto, Ribeiro, Ferreira, & Cunha, 2003). As a proinflammatory mediator, MPO is found in zyrophil granules in neutrophils and affects PMN superoxide production. After activation, neutrophils can degranulate and release MPO into the extracellular space, which can cause other tissue damage (Lau et al., 2005). Therefore, we selected the cytokine described previously as a measuring method for the response function of neutrophils in different stages of the germ-killing process. The CINC and MPO levels we measured after overtraining have a concordant rise in rat plasma, indicating that there is an enormous response from neutrophils.

**Association Between MPO Levels and Risk of Coronary Artery Disease**

Smith (2000) reported that circulating monocytes are activated by injury-related cytokines and in turn produce large quantities of proinflammatory IL-1$\beta$, IL-6, and/or TNF-$\alpha$, producing systemic inflammation. Elevated circulating cytokines coordinate the whole-body response, including immune response.

In addition, our previous study (Ru & Peijie, 2009) showed that overtraining enhances Th2-cell responses and induces a shift from Th1 to Th2 during the differentiation of naïve T-helper cells. This implies that the decrease in immune capacity and increase in infection rate postexercise are mainly related to the suppression of Th1 responses and less influenced by changes in humoral immunity. This explanation is consistent with a study (Lancaster & Halson, 2004) that showed that exercise could cause an imbalance in immunological regulation resulting in decreased cellular immunity.

**Excessive Oxidation and the Function of Neutrophils After Overtraining**

Neutrophils produce ROS, which assist in the clearance of damaged host tissue. However, our previous findings indicate that excessive exercise can lead to damage of DNA, lipids, and proteins indirectly (Dong et al., 2011). Alba et al. (2004) also found that ROS could lead to phagocytic cell death by interrupting tyrosine phosphorylation induced by the deactivation of AMPK in macrophages. Pyne, Smith, Baker, Telford, and

### Table 3 Changes of Hemoglobin, Cortisone, and Testosterone in Blood Plasma After 36 hr of Training ($M \pm SD, n = 8$ per Group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Hemoglobin, g/L</th>
<th>Cortisone, nmol/L</th>
<th>Testosterone, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>154.88 ± 0.23</td>
<td>227.23 ± 0.74</td>
<td>4.86 ± 0.46</td>
</tr>
<tr>
<td>Group E</td>
<td>134.00 ± 0.05**</td>
<td>134.89 ± 34.36**</td>
<td>2.17 ± 0.63**</td>
</tr>
</tbody>
</table>

*p < .05, **p < .01 compared with Group C.
Weidemann (2000) reported that neutrophil-derived ROS induced by exercise may overwhelm the body’s endogenous antioxidant defense mechanisms, and this can lead to oxidative stress.

To determine the oxidative-stress state in peripheral blood caused by ROS mediated by NADPH oxidase, we examined the plasma MDA level, the intercellular ROS, the activity of NADPH oxidase, the respiratory burst, and the function of phagocytosis of neutrophils in vitro after overtraining. As shown in Figures 3, 4, and 5, plasma MDA, intercellular ROS, and activity of NADPH oxidase in the overtraining Group E were higher than in rest Group C and Group D. However, there was no change in respiratory burst and phagocytosis of neutrophils in vitro after overtraining compared with the rest group. Moreover, the function of phagocytosis in Group D was decreased compared with Group E. This result indicates that overtraining can increase ROS generation and lead to oxidative stress in circulating blood. This peroxidation stress may damage neutrophils and lead to a decrease in both the respiratory burst and phagocytosis after overtraining in vitro. The DPI intervention can decrease the production of ROS mediated by NADPH oxidase and attenuate oxidative stress in blood.

Combined Effect of DPI Administration and Glutamine Supplementation on Rat Neutrophil Function Induced by Overtraining

As noted earlier, our studies showed that the respiration burst and phagocytosis function of neutrophils from both the single glutamine-supplementation group and DPI-administration group were reversed. This is the same response of neutrophil function as that studied by Lagranha et al. (2005). Furthermore, glutamine obviously reversed the decline of neutrophil function from the combined group. Lagranha et al. (2008) reported that glutamine administration caused a further increase in production of ROS (twofold) and in the production of reactive oxygen metabolites in neutrophils. It may be that glutamine supplementation with overtraining could lead to acute peroxidation stress and induce decreased function of neutrophils. It seems to conflict with the effect of DPI administration and glutamine supplementation on the neutrophil function induced by overtraining. However, overtraining is frequently associated with reduced availability of glutamine and decreased immunocompetence. Inactivity affects glutamine metabolism, but this subject was poorly investigated (Agostini & Biolo, 2010). The study of Lagranha et al. (2008) also demonstrated that acute exercise induced a marked decrease in nitric oxide production and the expression of inducible nitric oxide synthase. Moreover, recent findings from Lagranha’s groups (Lagranha, Hirabara, Curi, & Pithon-Curi, 2007; Lagranha et al., 2004) show that glutamine supplementation prevents exercise-induced neutrophil apoptosis and reduces p38 mitogen-activated protein kinase and JNK phosphorylation and caspase 3 expressions. New research on oxidative stress shows that discrete changes of activities of certain enzyme systems may be more important than the overall balance of production and removal of ROS. Such imbalance of nitric oxide and superoxide production could modify inflammation and immune regulation (Czesnikiewicz-Guzik et al., 2008).

Based on previously mentioned analysis, DPI administration may relieve the oxidative overstress with overtraining by cutting the pathway of production of ROS mediated by NADPH oxidase to prevent the inflammatory reaction and surrounding tissue damage. Furthermore, glutamine supplementation could augment the energy rate of neutrophils, inducible nitric oxide synthase activity, and prevention of neutrophil apoptosis induced by exercise. There may be an imbalance between NADPH oxidase and nitric oxide synthase in neutrophils that leads to the decline of neutrophil function during overtraining, so this exercise-induced imbalance could be modulated by combining the inhibitor of NADPH oxidase and glutamine supplementation.

Conclusion

The process of priming NADPH oxidase is a “double-edged sword,” as it contributes to a rapid and efficient elimination of the pathogens but can also induce peroxidation stress to damage the surrounding tissues. So NADPH oxidase priming and activation must be tightly regulated. We found that overtraining could activate NADPH oxidase and produce ROS in neutrophils; dysfunctional NADPH oxidase is responsible for superoxide anion production induced by overtraining. More important, our pilot study suggests that the excessive ROS generated by NADPH oxidase is related to the decline of neutrophil function induced by overtraining. Our study also shows the reverse effect of DPI administration and glutamine supplementation on the decline of rat neutrophil function after overtraining in vitro. These observations, although preliminary, may be of critical value to the modification of oxidative stress induced by overtraining, and they need to be further researched and characterized in a larger population.

Acknowledgments

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References


Reverse Neutrophil Function Induced by Overtraining


