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ORIGINAL RESEARCH

Effect of 6 months' training on the reactive oxygen species production capacity of neutrophils and serum opsonic activity in judoists

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ABSTRACT: The effects of long-term training on the production of reactive oxygen species (ROS) from neutrophils and serum opsonic activity (SOA) remain to date unknown. The aim of this study was to evaluate the effect of 6 months training on ROS production and SOA in judoists. Fifty-six judoists were enrolled this study. White blood cell counts, serum creatine kinase (CK), asparate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and ROS production from neutrophils, and serum opsonic activity (SOA) using the lucigenin and luminol probes, were measured before and after daily judo exercise (2 h) in March and September. The subjects started their training from March after no exercise for three months, and continued it for 6 months (until September). In March, myogenic enzymes such as CK, ASAT, LDH and neutrophil counts increased and immunoglobulins, complements and SOA decreased after daily judo exercise. Such significant changes were not seen in September. On the other hand, ROS significantly increased after daily judo exercise in both March and September, with no significant difference in the rates of change. In conclusion, 6 month training minimized the changes in SOA as well as muscle enzymes, neutrophil counts, serum immunoglobulins and complements. This could be categorized as a long-term training effect. However, no such change was seen in ROS. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: Long-term training; reactive oxygen species; serum opsonic activity; myogenic enzyme; neutrophil counts; judoist

INTRODUCTION

It has been reported that exercise of high intensity and frequency increased the tendency of athletes to catch cold (1–3), induced the 'over-training syndrome' and adversely affected the immune system. Furthermore, the non-specific immune system, particularly neutrophil function, is suppressed by intensive exercise training (1–3).

Although neutrophils play an important role in the first line of defence against invading microorganisms, the efficient clearance of microorganisms by neutrophils is mediated by opsonins such as immunoglobulins and complements. Serum opsonic activity (SOA) reflects the general biological activity of the humoral immune status, which finally contributes to the ability of neutrophils to ingest foreign bodies (phagocytosis) and to produce reactive oxygen species (ROS), which are powerful microbiocidal substances.

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Many studies have examined the relationship between neutrophil ROS production and exercise or short-term and long-term training (4–7). However, there are only a few studies on the simultaneous changes in ROS and SOA following acute exercise or training (7, 8) and there are no studies on these two parameters simultaneously in long-term training. One report has pointed out that these two parameters compensate for each other in acute or short-term exercise (9). Therefore, it is of considerable interest to ascertain whether the same phenomenon is also seen in long-term training.

The aim of this study was thus to examine changes in ROS production of neutrophils, SOA and other related parameters, including immunoglobulins and complements, brought about by long-term training.

MATERIALS AND METHODS

Subjects

The subjects in this study were 56 male judoists; 33 who entered the Nippon Sport Science University in March 2001, and 23 who entered the same university in March 2002. They all had a past history of practising judo for a

minimum period of 3 years, and had not participated in any judo exercise for at least the 3 month period preceding this study.

Seven students competed in the under-66 kg class, 13 in the under-73 kg class, 15 in the under-81 kg class, nine in the under-90 kg class, eight in the under-100 kg class and four in the 100 kg class. The mean age, body weight and height (± standard deviation) were 18.0 ± 0.1 years, 81.8 ± 13.5 kg and 172.1 ± 5.1 cm, respectively, at the beginning of this study.

Approval was obtained from the Ethics Committee of Hirosaki University School of Medicine. The study protocol and purpose were explained to, and informed consent was obtained from, all subjects before the study.

Survey period

The survey was performed twice: March (before the training period) and September (after 6 months' training) in 2001 and 2002, with pre-match weight reduction not being required during this period. All parameters were measured before (fasting condition) and immediately after daily judo exercise at each survey point.

The contents of the exercise menu are shown in Table 1. The training regimen consisted of exercises to strengthen physical fitness through running and weight training, and technical training for the judo matches. Training was performed for 6 days/week (one day was a free exercise day). Actual judo practice consisted of preparation for 15 min, actual practice (*Uchikomi*, such as throw-down, push-down and hook-down) for 20 min, *Randori* (mini-matches of 5 min each, repeated 10 times) for 100 min, and cooling down exercise for 15 min.

The daily judo exercise consisted of the usual 2 h practice, comprising preparation for 15 min, actual practice for 90 min (*Uchikomi* for 20 min, *Randori* for 70 min) and cooling down exercise for 15 min.

Table 1. Training programme per week during the research period

Day	06:30–07:30	09:00–11:30	17:30–20:00
Monday	Training A	Rest	Training D
Tuesday	Training B	Rest	Training D
Wednesday	Training C	Rest	Training D
Thursday	Training A	Rest	Training D
Friday	Training B	Rest	Training D
Saturday	Training C	Training D	Rest
Sunday	Rest	Rest	Rest

Training A: interval training consisting of sprint running (800 m × 1, 1400 m × 3, 200 m × 3, 100 m × 4) and jogging.
Training B: Weight training.
Training C: Distance running for 30 min and short sprint running (repeated sprints over 30–50 m).
Training D: Judo training practice.
Rest: Take a rest or attend lectures.

The mean pulse rate during all exercise sessions was measured for 10 judoists (Heart rate monitor, Polar Electic Co. Ltd, Finland), giving an average pulse rate of 128.8 ± 12.0/min. and a maximum pulse rate of 180.5 ± 14.0/min.

Body composition

As for body composition, body weight, relative percentage body fat (%fat) and fat-free mass (FFM) were measured using a body fat measurement apparatus (TBF-110, Tanita Co. Ltd., Tokyo).

Haematological parameters

Blood samples were collected before meals in the early morning to measure the following parameters: (a) white blood cell (WBC) and leukocyte counts in whole blood (using a blood cell autoanalyser, MicroBiff-II, Coulter Co., Ltd, California, USA); (b) lactate dehydrogenase (LDH), asparate aminotransferase (ASAT), alanine aminotransferase (ALAT) and creatine kinase (CK) (ultraviolet method used for each); (c) immunoglobulins (IgG IgA and IgM, nephrometry method); and (d) complements (C3 and C4, nephrometry method) in serum.

Measurement of ROS

Neutrophil oxidative burst was measured with two-colour flow cytometry. To measure the oxidative burst, hydroethidine (HE; 44.4 μmol/L; Polyscience Inc., Warrington, USA) was used as an indicator for the generation of oxygen free radicals. Hheparinized whole blood (100 μL) was mixed with 22 μL HE (final concentration, 8 μmol/L) and incubated at 37°C for 5 min. After the incubation, Lyse and Fix solution (IMMUNOTECH, Marseille, France) was added to lyse the red blood cells and to fix the samples. The samples were washed twice in phosphate-buffered saline with sodium azide (PBS^+), mixed with 50 μL paraformaldehyde (50 mg/mL; Wako Pure Chemical Industries Ltd, Osaka, Japan) and kept shaded and cool for 2 days.

Samples were analysed by flow cytometry (FACScan, Becton Dickinson, San Jose, USA) using CaliBRITE Beads (Becton Dickinson) as a control. For each sample, 10 000 neutrophils were analysed. The mean channel number of fluorescence intensity (FI) of activated neutrophils was used as the index of oxidative burst. The percentage of positive cells (%) was counted to express the rate of neutrophils producing reactive oxygen species (ROS). We calculated the cumulative fluorescence intensity (CFI), which is the value of FI multiplied by the ratio of positive cells. CFI was used as the quantitative index of total oxidative burst and total phagocytic activity.

Measurement of SOA

Preparation of serum sample. Serum samples were separated from blood, using Vacutainer blood-collection tubes (Becton Dickinson, Franklin Lake, USA), by centrifugation at 1000 × *g* for 10 min, after allowing the blood to clot for 30 min at room temperature. These samples were stored frozen at –80°C until analysis and samples were rapidly thawed at 37°C.

Opsonization of particles. Zymosan from *Saccharomyces cerevisiae*, a well-known activator of the alternative pathway of the complement system (10, 11), was employed for opsonized particles. Zymosan A (Sigma, USA) was suspended in Hanks' balanced salt solution (HBSS) at a concentration of 5 mg/mL and then opsonization was performed by adding the final concentration of 13% of serum samples and incubating at 37°C for 30 min. The particles were then washed twice with HBSS and resuspended to 5 mg/mL for lucigenin-dependent chemiluminescence (LgCL) and 1 mg/mL for luminol-dependent chemiluminescence (LmCL).

Chemiluminigenic probes. Two chemiluminigenic probes, lucigenin and luminol, were employed for the detection of ROS. Lucigenin was prepared by dissolving bis-N-methylacridinium nitrate (Sigma, USA) in HBSS to a final concentration of 0.5 mmol/L (pH 7.4). Luminol was prepared by dissolving 5-amino-2,3-dihydro-1,4-phatalazinedione (Sigma, USA) initially in 1 N NaOH to give a clear solution and then adjusted using HCl and HBSS to give a final concentration of 2 mmol/L (pH 7.4).

Isolation of neutrophils. Standard neutrophils were obtained from peripheral blood of a healthy adult male volunteer and separated by Histopaque density gradient centrifugation, according to the method already reported (12). Briefly, human blood was diluted with the same volume of HBSS containing heparin (5 U/mL) and this heparinized diluted blood was then settled onto Histopaque 1077 layered over Histopaque 1119 (Sigma, USA) and then centrifuged at 500 × *g* at 4°C for 30 min. The neutrophil rich layer was collected and washed twice in HBSS and the neutrophils were resuspended to 3.106 cell/ml using an automatic blood cell counter (Coulter MD II, Coulter Co. Ltd., Tokyo, Japan). Neutrophils were resuspended to 3×10^6 cells/mL using an automatic blood cell counter (Coulter MD II, Coulter Co. Ltd., Tokyo, Japan).

Measurement of chemiluminescence. Opsonized zymosan (OZ) suspension and chemiluminigenic probes prepared as above noted were added to each well of black flat-bottom microplates (Greiner Japan, Tokyo, Japan), and 50 μL standard neutrophils was added. The plates were automatically measured on the Lumi Box H-1000 (Maikurottekku Nichion, Funabashi, Japan) as described before (11). All measurements were performed at 37°C. The peak value in the response curve was expressed as peak height (PH) and the reaction time taken to reach PH was defined as peak time (PT). Each sample was run in duplicate and values were expressed as means.

Statistical analysis

Data were presented as means ± the standard deviation. The significance of changes was evaluated with a two-way ANOVA. Probability (p) values < 0.05 were considered to be significant.

RESULTS

Body composition

Both body weight and %fat significantly decreased in September ($p < 0.01$ for both parameters: Table 2).

Blood parameters

WBC counts and neutrophil counts (Table 3). WBC counts significantly increased after daily judo exercise in March ($p < 0.01$), but there was no significant change in September. Neutrophil counts significantly increased after daily judo exercise in both March and September ($p < 0.01$ in March and $p < 0.05$ in September). The increasing rates were significantly higher in March (45.7 ±

Table 2. Changes in height, body weight and body composition before and after 6 months of training

	March		September	
Item	Before exercise	After exercise	Before exercise	After exercise
Age (years)	18.0 ± 0.1	–	18.5 ± 0.5	–
Height (cm)	172.1 ± 5.1	–	172.2 ± 5.1	–
Body weight (kg)	81.8 ± 13.5	81.1 ± 13.4	79.7 ± 12.8††	79.4 ± 12.8
%Fat (%)	15.7 ± 4.0	–	14.4 ± 3.5††	–
Fat-free mass (kg)	68.5 ± 7.9	–	67.8 ± 8.0	–

††$p < 0.01$: significant difference from the value in March.

Table 3. Changes in WBC counts, neutrophil counts, immunoglobulins and complements

	March		September	
Parameter	Before exercise	After exercise	Before exercise	After exercise
WBC counts (/μL)	6314 ± 1343	9200 ± 2666**	6353 ± 1875	6536 ± 1464
Neutrophil counts (%)	48.0 ± 9.7	70.4 ± 8.7**	49.8 ± 8.4	56.9 ± 8.7*
IgG (mg/dL)	1235 ± 203	1197 ± 190*	1183 ± 194	1184 ± 189
IgA (mg/dL)	201.2 ± 65.3	194.6 ± 62.7*	193.2 ± 63.6	190.3 ± 60.3
IgM (mg/dL)	110.7 ± 44.2	106.5 ± 41.9	106.1 ± 39.2	103.9 ± 42.2
C3 (mg/dL)	106.2 ± 17.3	101.7 ± 16.1*	102.5 ± 15.7	100.2 ± 17.0
C4 (mg/dL)	22.7 ± 5.5	21.6 ± 5.2	21.3 ± 5.4	20.8 ± 5.3

*$p < 0.05$, **$p < 0.01$: significant difference between before and after the daily judo exercise.

Table 4. Changes in myogenic enzymes

	March		September	
Parameter	Before exercise	After exercise	Before exercise	After exercise
CK (U/I)	250.5 ± 177.0	376.9 ± 216.6*	326.3 ± 206.5	354.5 ± 229.6
ASAT (U/I)	23.2 ± 9.2	26.2 ± 8.3*	23.9 ± 6.9	25.3 ± 7.5
ALAT (U/I)	26.6 ± 14.8	26.8 ± 15.0	22.7 ± 12.9	22.8 ± 13.0
LDH (U/I)	281.1 ± 80.0	357.8 ± 118.6**	231.5 ± 48.2††	228.6 ± 37.3

*$p < 0.05$, **$p < 0.01$: significant difference between before and after the daily judo exercise.
††$p < 0.01$: significant difference from the value in March.

Table 5. Changes in reactive oxygen species

	March		September	
ROS	Before exercise	After exercise	Before exercise	After exercise
ROS production	177.2 ± 22.1	194.4 ± 30.1*	229.8 ± 58.2	265.7 ± 28.7**

*$p < 0.05$, **$p < 0.01$: significant difference between before and after the daily judo exercise.

21.1% for WBCs and 31.1 ± 14.6% for neutrophils) than in September (11.9 ± 11.1% for WBCs and 2.9 ± 18.9% for neutrophils; $p < 0.01$ for WBCs and $p < 0.05$ for neutrophils).

Immunoglobulins and complements (Table 3). Although IgG and IgA significantly decreased after exercise loading in March ($p < 0.05$ for each), such significant changes were not seen in September. IgM showed no significant change in either March or September.

Although C3 significantly decreased after daily judo exercise in March ($p < 0.05$), such a change was not seen in September. C4 showed no significant change in either March or September.

Myogenic enzymes (Table 4). Although CK, ASAT and LDH significantly increased after daily judo exercise in March ($p < 0.05$, $p < 0.05$, $p < 0.01$, respectively), such changes were not seen in September.

ROS

ROS significantly increased after daily judo exercise in both March and September ($p < 0.05$, $p < 0.01$, respectively; Table 5), with no significant difference in these rates of change (9.6 ± 21.9% in March and 15.7 ± 19.4% in September).

SOA

PH significantly decreased after daily judo exercise in both lucigenin and luminol in March ($p < 0.01$ in all). PT was significantly prolonged after daily judo exercise in both lucigenin and luminol in March ($p < 0.01$ in all). On the other hand, such changes in PH and PT in both lucigenin and luminol became smaller in September (Table 6).

DISCUSSION

Daily judo exercise increases the numbers of WBCs and neutrophils in an exercise loading-dependent manner (5, 6, 13–19). The mechanisms for this are thought to be as follows. Exercise increases the serum concentration of catecholamine and cortisol. Catecholamine washes out the neutrophils on the arterial endothelium, and cortisol mobilizes the neutrophils from bone marrow (7, 11, 20).

Table 6. Changes in serum opsonic activity

	March		September	
Parameter	Before exercise	After exercise	Before exercise	After exercise
LgCL PH ($\times 10^4$ cpm)	30.9 ± 9.5	25.4 ± 7.8**	27.9 ± 6.8††	28.2 ± 7.1
LgCL PT (s)	1085 ± 142	1196 ± 176**	1110 ± 115	1088 ± 101
LmCL PH ($\times 10^4$ cpm)	105.8 ± 13.3	99.1 ± 14.7**	104.0 ± 10.7	103.7 ± 11.3
LmCL PT (s)	1333 ± 120	1395 ± 145**	1365 ± 120	1320 ± 141

LgCL, lucigenin-dependent chemiluminescence; LmCL, luminol-dependent chemiluminescence; PH, peak height; PT, peak time; cpm, counts/min.
**$p < 0.01$: significant difference between before and after the daily judo exercise.
††$p < 0.01$: significant difference from the value in March.

Suzuki *et al.* reported that neutrophil counts due to these release mechanisms gradually disappeared over 3 days of daily repetition of exercise (21). They speculated that this may be because the systemic release of the above substances was attenuated. We think that the same mechanism induced the decrease in neutrophil counts in judoists after 6 months of training with daily judo exercise.

In order to evaluate muscle status, myogenic enzymes such as CK, ASAT and LDH were measured. Commonly accepted mechanisms behind the release of myogenic enzymes are damage to muscle tissue or changes in myocyte membrane permeability (4, 11). Accordingly, the significant increase post daily exercise in the levels of CK, ASAT and LDH were indicative of damage to the muscle tissue or changes in myocyte membrane permeability (6, 22–23). However, such significant changes were not seen in September. This was most probably due to the influence of the 6-month training regimen. Kuipers (24) reported that the well-trained subject showed a smaller increase in myogenic enzymes after exercise compared with subjects who had undergone no training. Therefore, in this study, the subjects' muscle tissues were strengthened by 6 months training, so the muscles were more resistant to daily judo exercise-related damage in September, even though the exercise loading was similar to March. In other words, although muscle weakness and muscle damage caused by daily judo exercise induced an increase in neutrophil counts in March, muscles strengthened by six months of training did not require any neutrophil mobilization.

Previous reports have also differed as to the changes in immunoglobulin caused by sports-related activities: an increase (10); a decrease (25, 26); and no change (16, 27–31). With regard to the relationship between exercising and complements, Dufaux *et al.* reported that the plasma concentrations of C3a and C4a, the degradation products of complements, increased after running for 2.5 h. They considered that muscle injury due to highly intensive exercising had triggered activation of the complement system (9). Therefore, a significant decrease in IgA, IgM and C3 after daily judo exercise, as observed in our current study, was assumed to have been caused by muscle injury brought about by highly intensive exercising. On the other hand, such significant increases were not seen after 6 months of training, probably due to the strengthened muscles, e.g. an increased resistance to damage brought about by the long-term training.

SOA contributes to the neutrophil bactericidal activity through accelerating the adhesion of neutrophils to opsonized substances via IgG, C3, etc., after which neutrophils engulf foreign bodies and produce ROS. Activated neutrophils initiate a 'respiratory burst' leading to the production of superoxide anions (O_2^-), which are quickly converted to hydrogen peroxide (H_2O_2). Neutrophil azurophilic granules contain large quantities of myeloperoxidase (MPO), which works in the presence of H_2O_2 and chloride ions to produce hydrochlorous acid (HOCl). HOCl has a significantly higher oxidizing potential than its precursors O_2^- and H_2O_2 and contributes to the complexity of the oxygen-dependent antimicrobial systems of neutrophils. In this study, we investigated the ROS metabolism using two chemiluminescence probes, i.e. lucigenin and luminol. Lucigenin is well-recognized as detecting O_2^-, whereas luminol mainly detects HOCl including the contribution of MPO degranulation.

In this study, acute exercise loading (daily judo exercise) induced a significant increase in ROS production and a significant decrease in SOA. The former change is associated with an increase in ROS hazards such as issue oxidation, ageing, etc., and the latter with a decrease in neutrophil function through a decrease in opsonic activity. We could also observe the 6 month training effect on SOA in this study; although SOA significantly decreased following daily judo exercise in March, such a change was not seen after the 6 month training regimen. This could be construed as the 6 month training regimen having a beneficial effect on the health of the subjects. On the other hand, such a training-related effect was not seen in ROS. However, ROS vary according to the extent of exercise loading as well as the timing of taking the samples. Previous studies to examine the ROS-producing activity of circulating neutrophils after exercise have thus reported conflicting findings, with increases (5, 23, 32–34), decreases (35–39), or no

change (25, 35, 36, 40) seen in the results of these studies. If the current subjects had performed a lesser exercise loading than the daily judo exercise loading in the current study, a long-term training effect on the ROS-producing capacity of neutrophils might have been seen.

On the other hand, some researchers have pointed out that ROS and SOA compensate for each other following acute exercise, with an inverse ratio change (9). However, no such change was seen in this study.

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