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ORIGINAL ARTICLE

Effect of chronic supplementation with shark liver oil on immune responses of exercise-trained rats

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Abstract Previous studies have reported that chronic supplementation with shark liver oil (SLO) improves immune response of lymphocyte, macrophage and neutrophil in animal models and humans. In a similar manner, exercise training also stimulates the immune system. However, we are not aware of any study about the association of exercise and SLO supplementation on immune response. Thus, our main goal was to investigate the effect of chronic supplementation with SLO on immune responses of exercise-trained rats. Male Wistar rats were divided into four groups: sedentary with no supplementation (SED, $n = 20$), sedentary with SLO supplementation (SEDslo, $n = 20$), exercised (EX, $n = 17$) and exercised supplemented with SLO (EXslo, $n = 19$). Rats swam for 6 weeks, 1.5 h/day, in water at 32 ± 1°C, with a load of 6.0% body weight attached to the thorax of rat. Animals were killed 48 h after the last exercise session. SLO supplementation did not change phagocytosis, lysosomal volume, superoxide anion and hydrogen peroxide production by peritoneal macrophages and blood neutrophils. Thymus and spleen lymphocyte proliferation were significantly higher in SEDslo, EX, and EXslo groups compared with SED group ($P < 0.05$). Gut-associated lymphocyte proliferation, on the other hand, was similar between the four experimental groups. Our findings show that SLO and EX indeed are able to increase lymphocyte proliferation, but their association did not induce further stimulation in the adaptive immune response and also did not modify innate immunity.

Keywords Shark liver oil · Supplementation · Immune responses · Physical exercise · Rat

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Introduction

Shark liver oil (SLO) has been traditionally used in Scandinavian medicine for the treatment of general debility and several other specific applications such as wound healing and the treatment of the respiratory and alimentary tracts (Pugliese et al. 1998; Krotkiewski et al. 2003). SLO is a rich source of a group of ether-linked glycerols known as alkylglycerols, which are found to a lesser extent in haematopoietic organs (e.g., bone marrow, spleen, liver), lymphatic tissues, blood and maternal milk (Hallgren and Larsson 1962). A number of observations have reported that these natural ether lipids have multiple biological activities, including haematopoiesis stimulation (Dulisch et al. 1985), tumor growth inhibition (Brohult et al. 1972; Pedrono et al. 2004a, b) as well as fertility and mobility improvement of spermatozoid (Cheminade et al. 2002). It has been previously reported that alkylglycerols significantly activate cytotoxic macrophages leading to enhanced phagocytosis (Berdel 1987). Also, alkylglycerols stimulate

neutrophil (Palmblad et al. 1990) and T and B lymphocyte functions (Homma and Yamamoto 1990; Hajimoradi et al. 2009).

Exercise intensity can be classified as mild, moderate and heavy, when their $\dot{V}O_{2\,max}$ correspond to <35%, 35–70% and >70%, respectively (Hackney 2006). It is well known that exercise training also induces several changes on immune system (Nieman 1994, 1997; Pedersen and Toft 2000; Bacurau et al. 2007; Leandro et al. 2007; Levada-Pires et al. 2007). These exercise training-induced changes in the immune responses can be influenced by multiple factors including mode, duration, and intensity of exercise (Nieman 1997) as well as hours and days after the last exercise session which are other important factors that might influence immune responses (Pedersen and Toft 2000). While previous studies have repeatedly demonstrated that immune functions are impaired after an acute bout of moderate/vigorous exercise (Pedersen and Toft 2000; Nguyen and Tidball 2003; Kwak 2006; Santos et al. 2007), others studies have shown that chronic moderate exercise improves immune functions (Tharp and Preuss 1991; Sugiura et al. 2001; Peijie et al. 2003; Levada-Pires et al. 2007). In fact, Sugiura et al. (2001) reported that phagocytic capacity and anion superoxide production by peritoneal macrophages were significantly increased in exercise-trained mice in comparison with sedentary mice following 12 weeks of moderate exercise training (15–120 min of treadmill running at a speed of 13 m/min in 5 days/week). Similarly, Tharp and Preuss (1991) showed that the proliferative response of spleen lymphocyte to concanavalin A (Con-A) or lipopolysaccharide (LPS) was significantly higher in moderate exercise-trained rats (45 min of treadmill running at a speed of 22 m/min in 5 days/week during 8 weeks). Taken together, these studies suggest that both innate and adaptive immunity are modified by moderate exercise training.

Despite the potential influence of the SLO supplementation and moderate exercise training on immune system, we are not aware of any study investigating the effect of association of SLO supplementation and exercise on immune responses. Therefore, our primary goal was to investigate the effect of chronic supplementation with SLO on immune responses of exercise-trained rats. Our hypothesis is that both factors diet and exercise will provide further increase on immune response.

Materials and methods

Study design

All procedures involving animals were approved by the Local Committee of Animal Welfare of the Federal University of Paraná. Male Wistar rats (aged 60 days) were maintained under controlled temperature (23°C), humidity, and 12/12 h light/dark cycle and were randomly divided into four groups: sedentary with no supplementation (SED, $n = 20$), sedentary with SLO supplementation (SEDslo, $n = 20$), exercised with no supplementation (EX, $n = 17$) and exercised with SLO supplementation (EXslo, $n = 19$). All diets contained the same amounts of protein (230 g/kg), fiber (60 g/kg), fat (40 g/kg), and vitamins and minerals (10 g/kg; Nuvital CR-1, Curitiba, Brazil). Two groups (SED and EX) were fed a regular chow diet, ad libitum, while the other two groups (SEDslo and EXslo) were fed a regular chow diet and orally supplemented with SLO (ECOMER®, kindly donated by Naturalis, São Paulo, Brazil). Each 250 mg of SLO contains 50 mg of alkylglycerols. The supplementation was at a level of 1 g/kg body weight per day and was provided as a single bolus daily using a micropipette. Body weight was monitored every 3 days.

Exercise training

Rats from the EX and EXslo groups exercised for 6 weeks (Rombaldi 1996; Moura et al. 2002) in swimming pool chambers at a water temperature of 32°C. The animals swam daily for 1 h and 30 min (three exercise bouts of 30 min, resting 5 min between each bout) carrying a load corresponding to 6% of their body weight. To avoid any potential acute effects of the exercise training, the animals were killed by decapitation without anesthesia 48 h after the last exercise session (Sugiura et al. 2001). Decapitation kills the animal rapidly and therefore prevents the increase of stressful hormones which would interfere on immune responses. Thymus, spleen, and gut-associated lymphoid tissues were removed for proliferation assay. Resident peritoneal macrophages and blood neutrophils were obtained for determination of phagocytosis, lysosomal volume, superoxide anion and hydrogen peroxide production.

Lymphocyte proliferation

Lymphocytes from thymus, spleen, and gut-associated lymphoid tissues were isolated and cultured at 37°C in an O_2:CO_2 (19:1) atmosphere in 96-well microtiter culture plates at a density of 5×10^5 cells/well and a total culture volume of 200 μL in RPMI buffer supplemented with 2 mM of glutamine, 10% fetal bovine serum, antibiotics (streptomycin and penicillin), and 5 μg/mL of concanavalin A (Con-A) or lipopolysaccharide (LPS). After 48 h of culture, 20 μL of [2-^{14}C]-thymidine was added to each well (0.01 μCi/well), and cells were incubated for another 18 h. Cells were then harvested onto glass fiber disks (Cox

Scientific, Kettering, England) and washed in a Skatron Cell Harvester (Skatron Instruments AS, Lierbeyen, Norway). Radioactive thymidine incorporation into DNA was determined by liquid scintillation counting in a Beckman LS 6000IC scintillation counter (Tharp and Preuss 1991; Nunes et al. 2008). Data is shown as lymphocyte proliferation in counts per minute (cpm).

Macrophage isolation

Resident macrophages were obtained by intraperitoneal lavage with 10 mL of sterile phosphate buffered saline (PBS). Peritoneal cells were collected by centrifugation (290 g at 4°C for 5 min), washed, and resuspended in PBS or RPMI medium after counting in a Neubauer chamber by optical microscopy by using a trypan blue solution (1%); viability was 96%. Peritoneal cells were characterized by flow cytometry; purity was about 50%. Macrophages were further purified by incubating peritoneal cells in tissue culture plates for 2 h and then washing three times with PBS to remove non-adherent cells (Bonatto et al. 2004).

Neutrophil isolation

Neutrophils were isolated from the blood of rats. Blood (10 ml) was diluted with an equal volume of PBS at pH 7.4 containing 100 mM $CaCl_2$–50 mM $MgCl_2$ and carefully layered on 10 ml of a commercial gradient of Lymphoprep (density = 1.077). The tube was centrifuged at 1,200 rpm at 4°C for 30 min. The supernatant, rich in mononuclear cells, was discarded. The pellet was submitted to hypotonic treatment with 10 ml of solution containing 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA to promote lysis of contaminated erythrocytes. The tube was homogenized and maintained for 10 min in ice to allow erythrocyte lysis. The tube was then centrifuged at 1,200 rpm at 4°C for 10 min. This procedure was repeated twice. Neutrophils were counted in a Neubauer chamber under optical microscope. The number of viable cells, always >95% neutrophils, was determined by trypan blue exclusion (Pithon-Curi et al. 2003).

Phagocytosis

Aliquots of peritoneal macrophage or blood neutrophil suspension (0.1 ml) were added to the wells of a 96-well flat-bottomed tissue culture plate (10^5 cells/well) and left to adhere for 40 min. Then 10 µl of neutral-red stained zymosan (1×10^8 particles/ml) were added to each well. After incubation for 30 min, the cells were fixed with Baker's formol–calcium (4% formaldehyde, 2% sodium chloride, 1% calcium acetate) for 30 min. The cells were then washed two times and centrifuged at 450 g for 5 min. The neutral-red stain was solubilized by adding 0.1 ml of acidified alcohol (10% acetic acid, 40% ethanol in distilled water) to each well. After 30 min, the absorbance of each well was read on a plate reader at 550 nm. Phagocytosis was calculated from a standard curve constructed from known amounts of stained zymosan and results expressed as absorbance/10^5 cells (Bonatto et al. 2004; Nunes et al. 2008).

Lysosomal volume

The uptake of the cationic dye neutral red, which concentrates in cell lysosomes, was used to assess the volume of the peritoneal macrophage or blood neutrophil lysosomal system. Twenty microliters of 3% neutral red in PBS were added to 0.1 ml of peritoneal macrophage or blood neutrophil suspension per microplate well for 30 min. The cells were then washed twice with PBS by centrifugation (450 g for 5 min). Neutral red was solubilized by a 30 min incubation adding 0.1 ml of 10% acetic acid plus 40% ethanol solution. The absorbance was read at 550 nm and lysosomal volume is expressed as absorbance/10^5 cells (Bonatto et al. 2004; Nunes et al. 2008).

Hydrogen peroxide production

Hydrogen peroxide production by peritoneal macrophage or blood neutrophil was measured as described by Pick and Mizel (1981). This assay is based on the horseradish peroxidase (HRPO)-dependent conversion of phenol red into a colored compound by H_2O_2. Macrophages or neutrophils (final volume 0.1 ml) were incubated in the presence of glucose (5 mM), phenol red solution (0.56 mM), and HRPO (8.5 U/ml) in the dark for 1 h at 20°C. After this period, absorbance was measured at 620 nm on a plate reader. The concentration of H_2O_2 was determined from a standard curve prepared in parallel. H_2O_2 production is expressed as absorbance/10^5 cells (Pizatto et al. 2006; Nunes et al. 2008).

Superoxide anion production

Superoxide anion production was estimated by the reduction of nitroblue tetrazolium (NBT) assay. Peritoneal macrophages or blood neutrophils (0.45 ml) suspended in PBS was incubated for 1 h at 37°C in the presence of 0.1% NBT. The reaction was stopped by adding 0.45 ml of acetic acid. Then the mixture was centrifuged for 30 s at 2,500 rpm. Reduction of NBT results in the formation of blue formazan which was detected spectrophotometrically at 560 nm. The results are expressed as absorbance/10^5 cells (Pizatto et al. 2006).

Determination of alkylglycerol profile by gas chromatography–mass spectrometry (GC–MS)

Total lipids were extracted from SLO capsules (Ecomer®) using chloroform–methanol (2:1 vol/vol) according to Folch et al. (1957). Then the content was hydrolyzed and kept under 100°C for 2 h. The samples were dried out under gas nitrogen and added 0.2 ml of ethanoyl ethanoate and 0.2 ml of pyridine followed by 30 min incubation at 100°C (Sassaki et al. 2008). Alkyglycerols were separated on a GC–MS Saturn 2000R using a CP-Sil-5 CB Chrompack® column (30 m × 0.25 mm) (Table 1).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by two-way analysis of variance by using SLO supplementation and exercise training as factors, followed by a post hoc Bonferroni test. A value for $P \leq 0.05$ was taken to indicate statistical significance.

Results

Body weight

Initial body weight was not different between the groups (data not shown). However, after 6 weeks of exercise training, body weight were significantly lower in exercise-trained rats (334.9 ± 8.2 and 353.6 ± 8.2 g for the EX and EXslo groups, respectively) when compared to sedentary rats (379.7 ± 12.8 and 383.8 ± 6.3 g for the SED and SEDslo groups, respectively) ($P < 0.05$).

Macrophage and neutrophil immune parameters

There was no difference between the groups in the phagocytosis, lysosomal volume, superoxide anion and hydrogen peroxide production ($P < 0.05$) by the peritoneal macrophages and blood neutrophils (Tables 2, 3, respectively).

Table 1 Percentage of alkylglycerol measured in the shark liver oil capsule

Alkylglycerol	% of alkylglycerol
Octadecenylglycerol (C18:1)	52.4
Hexadecylglycerol (C16:0)	17.0
Octadecylglycerol (C18:0)	8.4
Heptadecenylglycerol (C17:1)	1.3
Heptadecylglycerol (C17:0)	1.0
Others	19.9

Data are presented as mean ± SEM of 3 independent measurements

Lymphocyte proliferation

The proliferative response of gut-associated lymphocytes in the absence and presence of mitogens (Con-A or LPS) is presented in the Fig. 1. In the absence of stimuli, sedentary rats SLO supplemented increased significantly the lymphocyte proliferation when compared to sedentary ones ($P < 0.05$). Six weeks of exercise also caused an increase in the basal lymphocyte proliferation when compared to SED ($P < 0.05$). The association of exercise and SLO supplementation did not induce further increase in basal lymphocyte proliferation ($P > 0.05$ vs. SEDslo and EX). In the presence of Con-A, a T lymphocyte mitogen, gut-associated lymphocyte proliferation increased significantly by 3.6-, 2.1-, 2.1- and 2.3-fold in the SED, SEDslo, EX, and EXslo groups, respectively, when compared to absence of stimuli ($P < 0.05$). Upon stimulation with LPS, a B lymphocyte mitogen, the proliferative response of gut-associated lymphocytes was similar to basal condition in all experimental groups, except in the SED group where there was an increase by 2.5-fold ($P < 0.05$).

The proliferative response of spleen lymphocytes in the absence and presence of mitogens (Con-A or LPS) is presented in the Fig. 2. In the absence of stimuli, SEDslo, EX, and EXslo increased significantly ($P < 0.05$) the lymphocyte proliferation when compared to SED but was not different between them (SEDslo vs. EX and EXslo, $P < 0.05$). Con-A stimulation increased spleen lymphocyte proliferation by 2.6-, 3.1-, 2.1- and 2.4-fold in the SED, SEDslo, EX, and EXslo groups, respectively, when compared to basal proliferation ($P < 0.05$ vs. no stimuli). Sedentary SLO supplemented-Con-A lymphocytes (SEDslo) had a higher proliferation rate when compared to the other groups ($P < 0.05$). In the presence of LPS, spleen lymphocyte proliferation increased by 2.5-, 2.5-, and 3.2-fold in the SEDslo, EX, and EXslo groups, respectively ($P < 0.05$ vs. no stimuli) but did not change in the SED group ($P > 0.05$ vs. no stimuli). All groups LPS-stimulated presented a higher lymphocyte proliferation when compared to SED group ($P < 0.05$).

The thymus lymphocyte proliferation (Fig. 3), in the absence of stimuli, was higher ($P < 0.05$) only in the sedentary group SLO supplemented (SEDslo) when compared to SED. In the presence of Con-A there was an increase by threefold in SED, 2.5-fold in the SED_{SLO}, threefold in the EX, and 2.6-fold in the EX_{SLO} ($P < 0.05$ vs. no stimuli). Upon LPS stimulation SED, SED_{SL}, EX, and EX_{SLO} groups increased the proliferative response by 1.8-, 3.1-, 3.3- and 3.2-fold, respectively when compared to basal proliferation response ($P < 0.05$ vs. no stimuli). The lymphocyte proliferation in the presence of LPS was lower in the SED when compared to the other groups ($P < 0.05$).

Table 2 Phagocytosis of zymosan, lysosomal volume, superoxide anion and hydrogen peroxide production by peritoneal macrophages obtained from sedentary rats fed regular chow (SED), sedentary shark liver oil supplemented (SEDslo), exercised fed regular chow (EX), and exercised shark liver oil supplemented (EXslo) rats

Group	Phagocytosis (absorbance/10^5 cells)	Lysosomal volume (absorbance/10^5 cells)	Anion superoxide (absorbance/10^5 cells)	Hydrogen peroxide (absorbance/10^5 cells)
SED	0.521 ± 0.060	0.151 ± 0.012	0.411 ± 0.038	0.235 ± 0.023
SEDslo	0.455 ± 0.039	0.149 ± 0.010	0.327 ± 0.029	0.210 ± 0.024
EX	0.468 ± 0.047	0.173 ± 0.016	0.357 ± 0.035	0.237 ± 0.024
EXslo	0.446 ± 0.046	0.163 ± 0.017	0.331 ± 0.029	0.190 ± 0.024

Data are shown as Abs/10^5 cells mean ± SEM

Table 3 Phagocytosis of zymosan, lysosomal volume, superoxide anion and hydrogen peroxide production by blood neutrophils obtained from sedentary rats fed regular chow (SED), sedentary shark liver oil supplemented (SEDslo), exercised fed regular chow (EX), and exercised shark liver oil supplemented (EXslo) rats

Group	Phagocytosis (absorbance/10^5 cells)	Lysosomal volume (absorbance/10^5 cells)	Anion superoxide (absorbance/10^5 cells)	Hydrogen peroxide (absorbance/10^5 cells)
SED	0.413 ± 0.113	0.151 ± 0.008	0.318 ± 0.056	0.197 ± 0.038
SEDslo	0.434 ± 0.088	0.187 ± 0.011	0.261 ± 0.039	0.169 ± 0.033
EX	0.457 ± 0.085	0.214 ± 0.017	0.314 ± 0.041	0.160 ± 0.030
EXslo	0.520 ± 0.088	0.301 ± 0.039	0.267 ± 0.038	0.180 ± 0.030

Data are shown as Abs/10^5 cells mean ± SEM

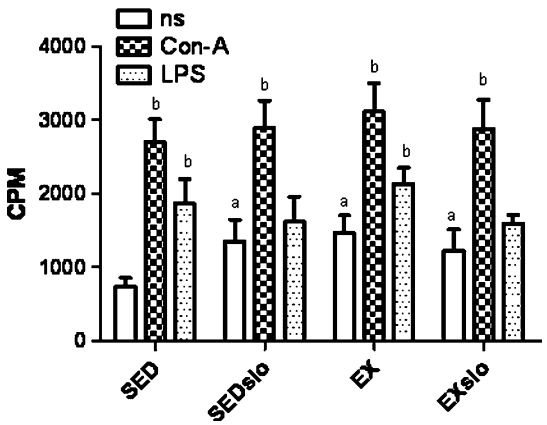

Fig. 1 Proliferation in counts per minute (*CPM*) of gut-associated lymphocytes obtained from sedentary rats fed regular chow (*SED*), sedentary shark liver oil supplemented (*SEDslo*), exercised fed regular chow (*EX*), and exercised shark liver oil supplemented (*EXslo*) rats after 66 h of culture in the absence (*ns*) or presence of concanavalin A (*Con-A*) or *LPS*. Data are shown as mean ± SEM. $^{a}P < 0.001$ versus control group in the absence of stimuli (*ns*); $^{b}P < 0.001$ versus when compared to its *ns* group

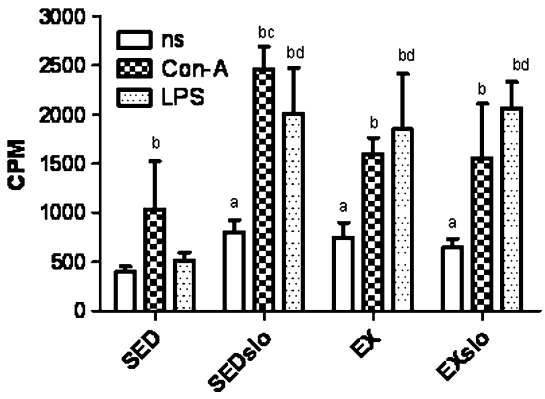

Fig. 2 Proliferation in counts per minute (*CPM*) of spleen lymphocytes obtained from sedentary rats fed regular chow (*SED*), sedentary shark liver oil supplemented (*SEDslo*), exercised fed regular chow (*EX*), and exercised shark liver oil supplemented (*EXslo*) rats after 66 h of culture in the absence (*ns*) or presence of concanavalin A (*Con-A*) or *LPS*. Data are shown as mean ± SEM. $^{a}P < 0.001$ versus control group in the absence of stimuli (*ns*); $^{b}P < 0.001$ versus when compared to its *ns* group; $^{c}P < 0.001$ versus all group Con-A stimulated; $^{d}P < 0.001$ versus control group LPS stimulated

Discussion

It is well documented that dietary fat and specific fatty acids induces changes in many components of the immune system (Calder 2001; Calder et al. 2002; Pizatto et al. 2006; Meksawan et al. 2004; Hill et al. 2007). Specifically, the chronic supplementation with SLO, a rich source of alkylglycerols, has been linked to an improved immune function, including increase in macrophage (Berdel 1987), neutrophil (Palmblad et al. 1990) and lymphocyte responses (Homma and Yamamoto 1990; Hajimoradi et al. 2009). In a similar manner, many studies have indicated that

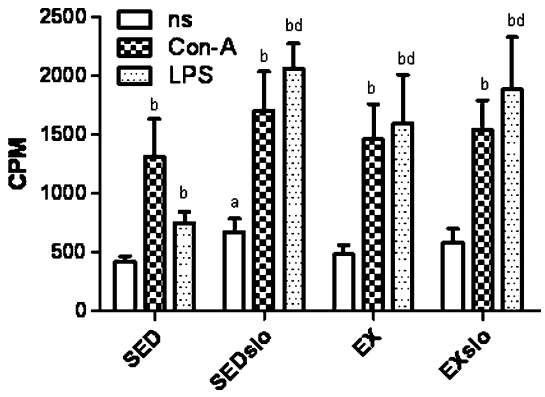

Fig. 3 Proliferation in counts per minute (*CPM*) of thymus lymphocytes obtained from sedentary rats fed regular chow (*SED*), sedentary shark liver oil supplemented (*SEDslo*), exercised fed regular chow (*EX*), and exercised shark liver oil supplemented (*EXslo*) rats after 66 h of culture in the absence (*ns*) or presence of concanavalin A (*Con-A*) or *LPS*. Data are shown as mean ± SEM. $^{a}P < 0.001$ versus control group in the absence of stimuli (*ns*); $^{b}P < 0.001$ versus when compared to its *ns* group. $^{c}P < 0.001$ versus control group LPS stimulated

exercise training also improves immune function (Nieman 1994, 1997; Bacurau et al. 2007; Leandro et al. 2007; Levada-Pires et al. 2007). However, studies have not been carried out to evaluate the combined effect of the SLO supplementation and exercise training on immune function. Therefore, the aim of the present study was to investigate the effect of chronic supplementation with SLO on immune responses from exercise-trained rats. The main finding of this study was that SLO supplementation, exercise and their association caused no effect on innate immune system, but did significantly increase the lymphocyte proliferation (adaptive immune system).

Macrophages play a critical role as first line of defense against microbial invaders by nature of their phagocytic, cytotoxic, and intracellular killing capacities (Adams and Hamilton 1984; Sugiura et al. 2001). In addition, macrophages are also important as antigen-presenting cells (Adams and Hamilton 1984). Neutrophils are the first barrier against infection due to their ability to migrate rapidly into loci of infection (Levada-Pires et al. 2007). Although previous studies have reported that the direct stimulation by SLO or indirect stimulation by alkylglycerols may also modify both neutrophils and macrophages function (Palmblad et al. 1990; Lewkowicz et al. 2005), the results of our study demonstrate that the chronic supplementation with SLO was not able to alter the phagocytic capacity, lysosomal volume, and production of superoxide anion and hydrogen peroxide by peritoneal macrophages and blood neutrophils from sedentary and exercise-trained rats (Tables 1, 2). These results are not in agreement with other studies that demonstrated an improved macrophage and neutrophil function after stimulation by alkylglycerol, an ether lipid compound found in SLO (Yamamoto et al. 1988; Yamamoto and Ngwenya 1987; Homma and Yamamoto 1990). We believed that these controversial findings can be partly explained by the different approaches used. For example, Yamamoto et al. (Yamamoto et al. 1988; Yamamoto and Ngwenya 1987; Homma and Yamamoto 1990) investigated in vitro the activities of resident peritoneal macrophages and blood neutrophils. We, on the other hand, investigated ex vivo the activities of these immune cells. Therefore, in vitro and ex vivo approaches might be the reason for these different findings.

Lymphocytes are the primary components of the adaptive immune system and comprise T and B lymphocytes (Pedersen and Toft 2000). Determination of the proliferative response of lymphocytes on stimulation with various mitogens in vitro is a well-established assay to examine the functional capacity of T and B lymphocytes (Nieman 1997). We investigate the lymphocyte proliferation from lymphocytes obtained from secondary lymphoid organs (thymus, spleen and lymph node) to check whether adaptive immune response might be modulated by exercise training and/or possibly by SLO supplementation in a different fashion (Hoffman-Goetz et al. 1989; Ferry et al. 1991). First, we demonstrate that lymphocytes basal proliferation rate was increased by SLO supplementation and exercise, which can be interesting. Then, when a mitogen challenge was added to culture medium, cells proliferate even more. Moreover, gut-associated lymphocyte proliferation was similar between experimental groups, but in the thymus and spleen the lymphocyte proliferation was significantly different between these experimental groups. Therefore, lymphocytes obtained from different compartments possibly contribute to the controversial results regarding exercise training and lymphocyte functions (Hoffman-Goetz et al. 1989; Ferry et al. 1991; Tharp and Preuss 1991; Peijie et al. 2003; Kwak 2006; Santos et al. 2007). These findings and their differences lead us to advise that results regarding immune system using lymphocytes from different lymphoid organs must be carefully interpreted as a response of the whole immune system.

In agreement with previous studies (Homma and Yamamoto 1990; Tharp and Preuss 1991; Pedersen and Toft 2000; Peijie et al. 2003; Pedrono et al. 2004b), the present results demonstrated that chronic supplementation with SLO and moderate exercise training did improve thymus and spleen lymphocyte proliferation in rats, except to gut-associated lymphocyte proliferation. However, no synergistic effect of combined SLO supplementation and moderate exercise training on proliferative response of lymphocytes was observed. This suggests that both agents (exercise and SLO) might share the same cell-signaling pathways to activate lymphocyte proliferation. Further studies must be carried out to test this hypothesis. Another

explanation for these results might be duration and or dose of supplementation or both. This hypothesis must be tested.

In summary, our results show that chronic supplementation with SLO has no effect on innate immunity from control or exercise-trained rats. On the other hand, SLO and moderate exercise intensity (average lactate concentration $\sim$4.8 mmol L^{-1}) are positive immunomodulators, but their association did not induce any further increase in the adaptive immune response.

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