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Research Article

Effect of different loads of treadmill exercise on Th1/Th2 cytokine balance in rat splenocytes

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ABSTRACT

The effect of moderate and overtraining exercise on Th1/Th2 balance was evaluated in rat splenocytes. Male Wistar rats were divided into sedentary control (C), moderately trained (MT; V = 20 m/min, 30 min/day, 8 weeks), overtrained (OT; V = 25 m/min, 60 min/day, 11 weeks) and recovered after overtraining (OR) (OT plus 2 weeks recovery) groups. At the end of study, cell viability, proliferation, interleukin 4 (IL-4) and interferon-γ (IFN-γ) secretion were evaluated in non-stimulated, phytohemagglutinin (PHA) and concavaline A (Con A)-stimulated splenocytes. Cell viability increased in MT and OR groups compared to control. Cell proliferation was higher in OR group than other groups. IL-4 concentration in PHA-stimulated cells from MT and OT groups, and IL-4 concentration in Con A-stimulated cells from OR and OT groups, were higher than the control group, but not for IFN-γ. In non-stimulated cells, IFN-γ/ IL-4 ratio was higher than MT and OT groups. In PHA and Con A-stimulated cells, IFN-γ/ IL-4 ratio was lower in exercise groups than control. We previously showed that moderate exercise increases Th1 cytokines in serum, but in splenocytes, Th2 or Th1 response may increase depending on the type of mitogen stimulation. Two-week recovery restored Th1/Th2 balance, only in non-stimulated splenocytes of overtrained animals.

Introduction

It has been shown that both physical and psychological stressors may affect the immune system in a similar way as that of the neuroendocrine system (Wallberg et al., 2011). The level of physical activity as one of the most important components of an individual's behavior and lifestyle may change the set point of the immune system (Karsten et al., 2016). In the past two decades, most investigations revealed that regular exercise with moderate intensity balances the immune system and decreases the risk of non-communicable chronic and infectious diseases in human population compared to physical inactivity (Pimlott, 2010). In addition, regular exercise can slow down the inflammation-induced damage and aging in the cells (Silva et al., 2016). However, overtraining may lead to imbalanced immune system and increased risk of inflammation in the same way (final impact on the immune function) as that caused by a sedentary lifestyle (Gholamnezhad et al., 2014b). Several studies have reported that the effect of exercise on the immune system is probably dependent on the nature, intensity and time delay between training bouts as well as immune parameter (Th1/Th2 balance as well as pro and anti-inflammatory cytokines) (Cox et al., 2007; Karsten et al., 2016; Ru and Peijie, 2009). Exercise can regulate both humoral and cellular aspects of the immune system and change the dynamic equilibrium between pro- and anti-inflammatory cytokines (Gholamnezhad et al., 2014b). These changes may be caused by modulating in metabolic signals and stress hormones levels and releasing adipokines and cytokines from non-immune targets such as muscle and adipose tissues (Lira et al., 2010).

Cytokines secreted by T cells play an important role in the adaptive immune responses (Dinarello, 1998). Intracellular pathogens begin a cellular response, which induces differentiation of naive CD4 + T helper cells to type 1 T helper (Th1). On the other

Abbreviations: IL-4, interleukin 4; IFN-γ, interferon-γ; PHA, phytohemagglutinin; Con A, concavaline A; MNCs, mononuclear cells; TNF-α, tumor necrosis factor alpha; NO, nitric oxide; WST, water-soluble tetrazolium salts; BrdU, bromodeoxyuridine.

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hand, extra-cellular pathogens initiate a humoral response and induce differentiation of naive CD4 + T helper cells to type 2 T helper (Th2) (Mosmann et al., 2005). Th1 lymphocytes are cell-mediated immune effectors which produce interferon γ (IFNγ), interleukin 2 (IL-2) and tumor necrosis factor-β. Whereas, Th2 lymphocytes combat extracellular pathogens by development of humoral immunity and producing cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13 (Abbas et al., 2012; Seder and Paul, 1994). These two pathways have cross-regulatory signaling. The ratio of IFNγ (Th1 prototype cytokine) and IL4 (Th2 prototype cytokine) is regarded as a common indicator of Th1/Th2 balance, which is a marker of immune system changes (Zhao et al., 2012).

Although the effect of post-exercise immunomodulation in Olympic and marathon race athletes was evaluated, the impact of different levels of exercise on immune system components had not been well determined. In competitive training, excessive physical activity in combination with inadequate recovery can lead to overreaching and possibly overtraining. The imbalance between training period and recovery may lead to functional overreaching (FOR), non-functional overreaching (NFOR) or overtraining syndrome (OTS) (Meeusen et al., 2006). In overtraining, a wide range of hormonal, metabolic, and immunologic factors which are crucial for homeostatic balance is altered (Gholamnezhad et al., 2014b; Hohl et al., 2009; Ogonovszky et al., 2005; Radak et al., 2008). A significant correlation between lactate levels during severs exercise training and muscle fatigue had been suggested. The rapid increase in blood and muscle lactate (due to tissue hypoxia) can lead to muscular fatigue, sympathetic stimulation and stress hormones (glucocorticoids and catecholamines) elevation (Kristensen et al., 2005). Overtraining-induced imbalanced immune system may result in increased incidence of viral infection and chronic low grade inflammation, which had been reported by champion athletes after severe training without enough recovery (Gleeson and Williams, 2013; Lancaster et al., 2004). However, moderate exercise changes Th1/Th2 balance toward Th1 pathway (Zhao et al., 2012). Other beneficial immune changes observed after moderate exercise are increased number of NK cells (Jankowsky et al., 2005), enhanced phagocytosis and macrophages function (Ortega et al., 2007) as well as IL-6-mediated functions such as reduction of adipokines (leptin and lipocalin-2), and cytokines (TNF-α, IL-6 and IL-18) production (Eyre and Baune, 2012; Petersen and Pedersen, 2005). However, high-intensity exercise could reduce the number of NK cells, which causes Th1/Th2 imbalance and may suppress antigen processing by macrophages (Ceddia and Woods, 1999; Ortega et al., 2007; Ru and Peijie, 2009). Previously, we showed that overtraining suppresses the immune system (Gholamnezhad et al., 2014b). Also, significant elevations in serum IL-6/IL-10 (following both moderate and overtraining exercise) and TNFα/IL-10 ratio (only after overtraining) were observed, which may indicate the effect of exercise on pro/anti-inflammatory cytokine balance is load-dependent (Gholamnezhad et al., 2014a). In addition, our findings indicated that serum IFN-γ/ IL-4 ratio increases in animals with moderate exercise while it decreases in overtraining group. After overtraining, prolonged Th2 domination and chronic low-grade inflammation may make athletes susceptible to viral infections, asthma, allergy, autoimmune diseases and cancer (Gleeson and Williams, 2013; Kaiko et al., 2008). There are not enough evidences about other markers and changes in immune system regulatory pathways after this immunosuppression. Evaluation of isolated immune cell response *in vitro* and *ex vivo* may explain the immune system changes and related illness after long term sever exercise (Peake et al., 2017). It is not clear whether the spleen lymphocytes or other components of the immune system are responsible for these changes. Moreover, the response of splenocytes isolated from exercised and sedentary animals, may alter after being activated by an antigen or a mitogen. Therefore, in the present study, the immunomodulatory effects of moderate and overtraining exercises were evaluated at the cellular level, by investigation of the response of rat splenocytes to different mitogens.

Table 1
Moderately training protocol.

Experimental week	Training speed (m/min)	Training time (min)	Number of Days/week
0 = familiarizing	12	10	5
1	15	20	6
2	20	30	6
3	20	30	6
4	20	30	6
5–8	20	30	6

Materials and methods

Animals

Adult male Wistar rats (6–8 weeks old; 180–200 g) were kept at animal house of School of Medicine, Mashhad University of Medical Sciences (Mashhad, Iran). Animals were housed under environmentally controlled conditions (12 h/12 h light/dark cycle; 22–24 °C) and had free access to food and water *ad libitum* throughout the experiment. The experimental protocols were according to guidelines of animal studies and approved by the Animal Experimentation Ethics Committee of Mashhad University of Medical Sciences (Mashhad, Iran).

Training protocol

A motorized treadmill with 4 individual lanes was used. A shock grid at the back of the treadmill provided a mild shock (0.5 mA, 1 Hz) if the rat's pace went below the treadmill rate during familiarizing period, as recommended by a previous study (Hohl et al., 2009); it was shown that this shock was not interfering with the measurements (Dao et al., 2013). The animals underwent a familiarizing period for one week before the beginning of the experiments. They were placed on the treadmill 5 days for 10 min/day at a speed of 12 m/min at 0% degree inclination. Then, they were scored 1–5 depending on running quality and rats that ran voluntarily with mean rating 3 or higher (n = 24) were separated from those refused to run (n = 6) and chosen for the study. This procedure was used to exclude possible different levels of stress among animals (Hohl et al., 2009).

Next, the 24 rats were randomly divided into four equal groups including: sedentary control (C), moderately trained (MT), overtrained (OT) and recovered after overtraining (OR). The animals of the control group were placed on the treadmill to experience the stress of treadmill training. Exercised groups undertook a progressive load of training 6 days a week to enhance cardiorespiratory fitness and a 5-minute warm-up and cool-down were done during each session. Moderately trained groups underwent 8-week exercise at a speed of 15 m/min for 20 min, 6 days/week but the intensity of exercise was increased to 20 m/min for 30 min at the onset of the second week (Table 1) (Kim et al., 2003). OT and OR groups were submitted to a 3-phase program (Table 2). In the first 4 weeks (phase I), the training speed increased from 15 to 25 m/min and training time increased from 20 to 60 min. In the second 4 weeks (phase II), training load was similar to that of phase I. During the last 3 weeks (phase III), running intensity and training duration remained unchanged, but the number of daily exercise bouts was increased to 2, 3, and 4 times where the recovery time (animal rest) between training sessions was 4, 3 and 2 h, respectively. The OR group had a 2-week recovery period after the last exercise session

Table 2
Overtraining protocol.

Experimental week	Training phase	Training speed (m/min)	Training time (min)	Number of daily session	Recovery between session (h)
0 = familiarizing	I	12	10	1	24
1	I	15	20	1	24
2	I	20	30	1	24
3	I	22.5	45	1	24
4	I	25	60	1	24
5–8	II	25	60	1	24
9	III	25	60	2	4
10	III	25	60	3	3
11	III	25	60	4	2

Table 3
Performance test (PT, kg·m) analysis in Moderate trained (MT), Overtrained (OT), Recovered after overtraining (OR) groups (for each group, n = 6).

Week	PT	MT	OT	OR
0	1	174.60 ± 13.31	132.97 ± 18.06	139.52 ± 17.19
2	2	264.09 ± 36.63	$308.88 \pm 32.27^{**}$	$278.10 \pm 26.52^{*}$
4	3	333.93 ± 45.06	$445.09 \pm 42.13^{***}$	$448.90 \pm 46.91^{***}$
8	4	425.70 ± 36.40	$331.32 \pm 37.80^{***}$	$361.19 \pm 28.35^{***}$
9	5	–	$254.20 \pm 32.86^{++}$	$222.50 \pm 26.44^{+++}$
10	6	–	$193.56 \pm 30.36^{+++}$	$122.74 \pm 19.90^{+++}$
11	7		$146.10 \pm 16.95^{+++}$	$107.46 \pm 17.18^{+++}$
13	8			$113.29 \pm 14.21^{+++}$

Values are presented as mean ± SEM.
Statistical differences between PT1 and PT2, PT3, PT4: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
Statistical differences between PT4 and PT5, PT6, PT7, PT8: $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$.
Data of each test in groups was compared with PT1 or PT4 using repeated measurements.

(Hohl et al., 2009). The training program was evaluated by a performance test (for determination of maximal effort, the treadmill speed increased gradually until animal refused to run) at the end of each phase. For evaluation of overload and fatigue time for each rat, a performance test was done at the end of each training section (Table 3). The performances test were performed at the end of animals familiarization period (week 0), the second, fourth and 8 weeks after training. For OT protocol three more tests were done at the end of the ninth, 10 and 11 week. An additional test was performed after two weeks recovery in OR group.

For performance quantification, a mass model (mass × speed stage × minutes performed at each stage) was used (Hohl et al., 2009). In this study, a standard training protocol for overtraining without enough recovery time (an imbalance period between exercise bouts and rest) was used. This protocol caused a significant decline in performance (overtraining marker) as shown in previous studies (Gholamnezhad et al., 2014a; Hohl et al., 2009).

Isolation of splenocytes

Splenocytes were isolated according to a previously described method (Asea and Stein-Streilein, 1998). Spleens were aseptically removed from rats (n = 6) and homogenized in complete RPMI-1640 medium (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate and HEPES buffer). Cell suspension was filtered through 70 μm nylon cell strainers (BD, USA) and cells were collected following centrifugation. Red blood cells were lysed using Tris-ammonium chloride lysis buffer (0.16 M NH_4Cl and 0.17 M Tris-HCl) and after washing them twice, splenocytes were finally re-suspended in complete RPMI-1640 medium and cell viability was determined using trypan blue dye (0.4% w/v) exclusion, revealing > 98% viability. All cell culture reagents were purchased from Gibco® (Invitrogen, Waltham, USA).

Experimental groups

Three main sub-groups of cells were designated for each group: non-stimulated splenocytes, PHA (5 μg/ml)-stimulated splenocytes, and Con A (2.5 μg/ml)-stimulated splenocytes. In this study, PHA as a selective and non-specific T cell mitogen and Con A as a specific T cell mitogen, were used (Perrin et al., 1997).

In vitro *splenocytes viability assay*

Cell viability was evaluated using water-soluble tetrazolium salts (WST-1) colorimetric assay kit (Roche Diagnostic, Mannheim, Germany) based on the manufacturer's instructions. Briefly, splenocytes were suspended in complete RPMI-1640 medium and seeded at 10^5cell/200 μl per well (triplicates) in flat-bottom 96-well culture plates and treated as described above. After 48 h, cells were incubated with 10 μl/well WST-1 reagents for 3 h. Then, the optical density was measured by a microplate reader (Biotek, Winooski, USA) at 450 nm with a reference at 630 nm.

In vitro *splenocytes proliferation assay*

Splenocytes proliferation was examined using cell proliferation bromodeoxyuridine (BrdU) ELISA (colorimetric) kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Briefly, splenocytes were suspended in complete RPMI-1640 medium and seeded at 10^5cell/200 μl per well (triplicates) in flat-bottom 96-well culture plates and treated as explained above. After 30 h, 20 μl of BrdU labeling solution was added to each well, cells were re-incubated for 18 h and the procedure was done according to the instructions. The optical density was measured by a microplate reader (Biotek, Winooski, USA) at 450 nm with a reference at 630 nm.

Cytokine assays

Splenocytes were suspended in complete RPMI-1640 medium and seeded at 10^6cell/1000 μl per well (duplicates) in flat-bottom 24-well culture plates and treated as mentioned above. After 48 h, the supernatant was collected and immediately stored at −20 °C until analyzed. Cytokine determination was performed with commercially platinum ELISA kits (Bender Med system, Austria) based on the manufacturer's instructions. Kits were carefully checked for specificity, sensitivity, and reliability. Serum concentrations of IL-4 and IFNγ were measured using rat ELISA kits, BMS628 and BMS621, respectively. The absorbance was measured using a spectrophotometer and a microplate reader (Biotek, Winooski, USA) and the concentration of each cytokine was calculated using a comparison curve plotted following the same measurement using GraphPad Prism 5. For checking the reliability of colorimetric assay, Biotek company test plates were used for calibration of microplate readers. Each cytokine assay was performed in duplicate, each time. The ratio of IFNγ (Th1 prototype cytokine) to IL4 (Th2 prototype cytokine) was determined in supernatant sample for each animal splenocytes.

Statistical analysis

The results were presented as mean ± SEM. Group-data comparisons (more than two groups) were performed using a one way analysis of variance (ANOVA) with Tukey-Kramer post-tests. Group-data comparisons (two groups) were performed using

unpaired t-test. Comparison within groups (tests in different time tables) was done using repeated measures analysis of variance. A value of $P< 0.05$ was accepted as statistically significant.

Results

Comparisons of performance among groups

The MT animals showed a significant increase ($P < 0.05$ to $P < 0.001$) in performance in tests 2–4 compared to test 1. In OT and OR groups, animal performance increased ($P < 0.05$ to $P < 0.001$) during the first 8 weeks, but increasing training load caused a significant decline ($P < 0.01$ to $P < 0.001$) in the next three weeks. Even after a 2-week recovery in OR group, performance was lower ($P < 0.001$) than pre-exercise value (Table 3).

Comparison of stimulated and non-stimulated splenocytes viability in different exercised groups

In non-stimulated splenocytes, cell viability in MT and OR groups were significantly higher than control group ($P < 0.01$ and $P < 0.001$, respectively). In addition, cell viability in OR group was higher than that of MT and OT groups ($P < 0.001$) (Fig. 1a).

In PHA-stimulated splenocytes, cell viability in MT and OR groups were significantly higher than that of control group ($P < 0.001$ for both groups). Cell viability in OT group was lower than that of MT group ($P < 0.01$) and in OR group was higher than those of MT and OT groups ($P < 0.001$ for both groups) (Fig. 1b).

In Con A-stimulated splenocytes, cell viability in MT and OR groups were significantly higher than that of control group ($P < 0.001$ for both groups). Cell viability in OT group was lower than those of MT and OR groups ($P < 0.001$ for both groups) (Fig. 1c).

Comparison of stimulated and non-stimulated splenocytes proliferation in different exercised groups

In non-stimulated splenocytes, cell proliferation in OR groups was significantly higher compared to control ($P < 0.001$), MT ($P < 0.001$) and OT group ($P < 0.05$) (Fig. 2a).

In PHA-stimulated splenocytes, cell proliferation in MT group was lower ($P < 0.05$) than control group and in OR group was higher than MT and OT groups ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 2b).

In Con A-stimulated splenocytes, cell proliferation in OR group was higher than MT group ($P < 0.01$) (Fig. 2c).

Comparison of stimulated and non-stimulated splenocytes cytokine secretion in different exercised groups

In non-stimulated splenocytes, there was no significant difference in terms of IL-4 secretion among different groups (Fig. 3a). The IFNγ secretion in OR group was higher than that of MT group ($P < 0.05$), but there were no significant differences among other groups (Fig. 4a). In OR group, the IFNγ/IL-4 ratio was significantly higher than that of MT ($P < 0.05$) and OT ($P < 0.01$) groups (Fig. 5a).

In PHA-stimulated splenocytes, IL-4 secretion in MT and OT groups was higher compared to control group ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3b). IL-4 secretion in OR group was lower than those of MT and OT group ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 3b). However, the IFNγ/IL-4 ratio in OT and OR groups was lower ($P < 0.01$ for both groups) and in MT group was higher ($P < 0.05$) compared to control group (Fig. 5b). In addition, the IFNγ/IL-4 ratio in MT group was higher than those of OT and OR groups ($P < 0.001$ for both groups) (Fig. 5b).

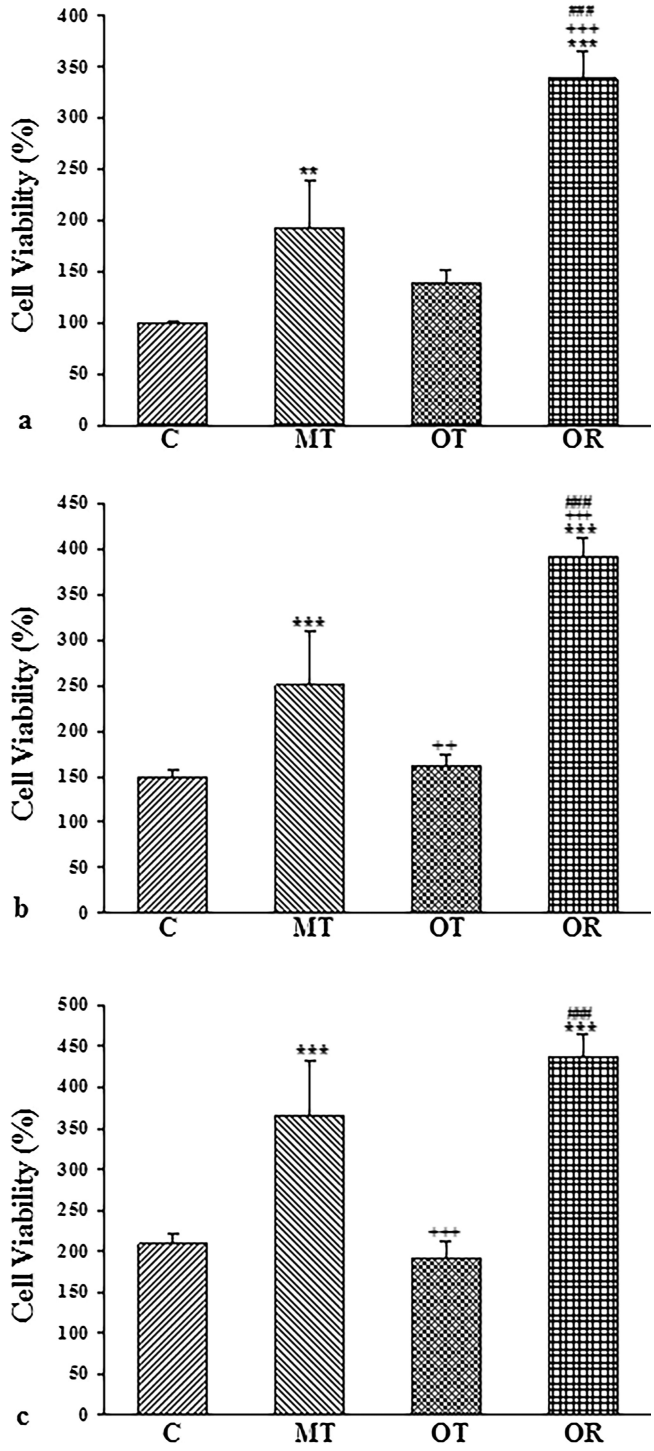

Fig. 1. Percentage of cell viability of non-stimulated (a), PHA (b), and Con A (c)-stimulated splenocytes. The groups presented as sedentary control rats (C), moderately trained (MT), overtrained (OT), recovered after overtraining (OR), (for each group, $n = 6$). Data are mean ± SEM values. ** $P < 0.01$, *** $P < 0.001$; compared to group C. $^{++}$ $P < 0.01$, $^{+++}$ $P < 0.001$; compared to group MT. $^{###}$ $P < 0.001$; compared to group OT.

In Con A-stimulated splenocytes, IL-4 secretion in OT and OR groups was higher than that of control group ($P < 0.001$ and $P < 0.01$, respectively). IL-4 secretion in OT group was also higher than MT groups ($P < 0.05$) (Fig. 3c). There were no significant differences in terms of IFNγ secretion among different groups (Fig. 4c). In addition, the IFNγ/IL-4 ratio in MT, OT and OR groups was lower than that of control group ($P < 0.01$ to $P < 0.001$) (Fig. 5c).

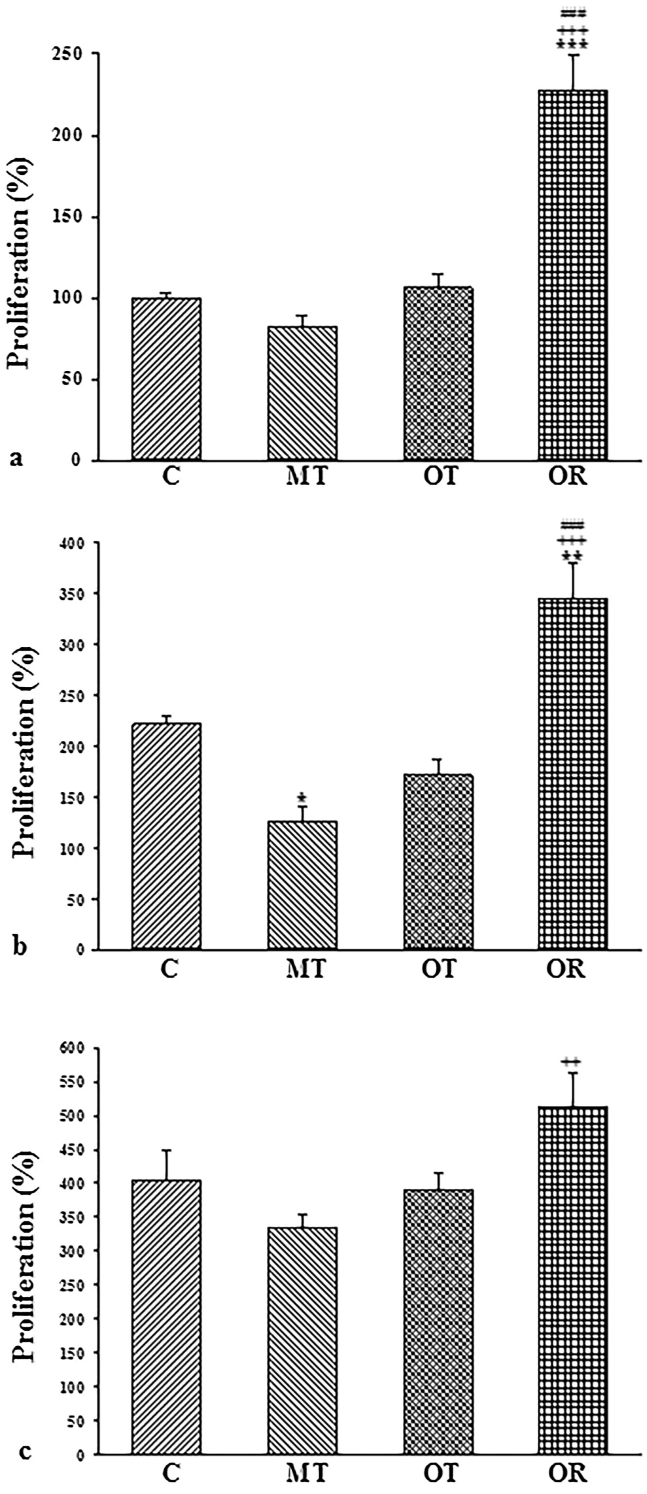

Fig. 2. Percentage of proliferation of non-stimulated (a), PHA (b), and Con A (c)-stimulated splenocytes. The groups presented as sedentary control rats (C), moderately trained (MT), overtrained (OT), recovered after overtraining (OR) (for each group, n = 6). Data are mean ± SEM values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; compared to group C. $^{++}P < 0.01$, $^{+++}P < 0.001$; compared to group MT. $^{\#\#\#}P < 0.001$; compared to group OT.

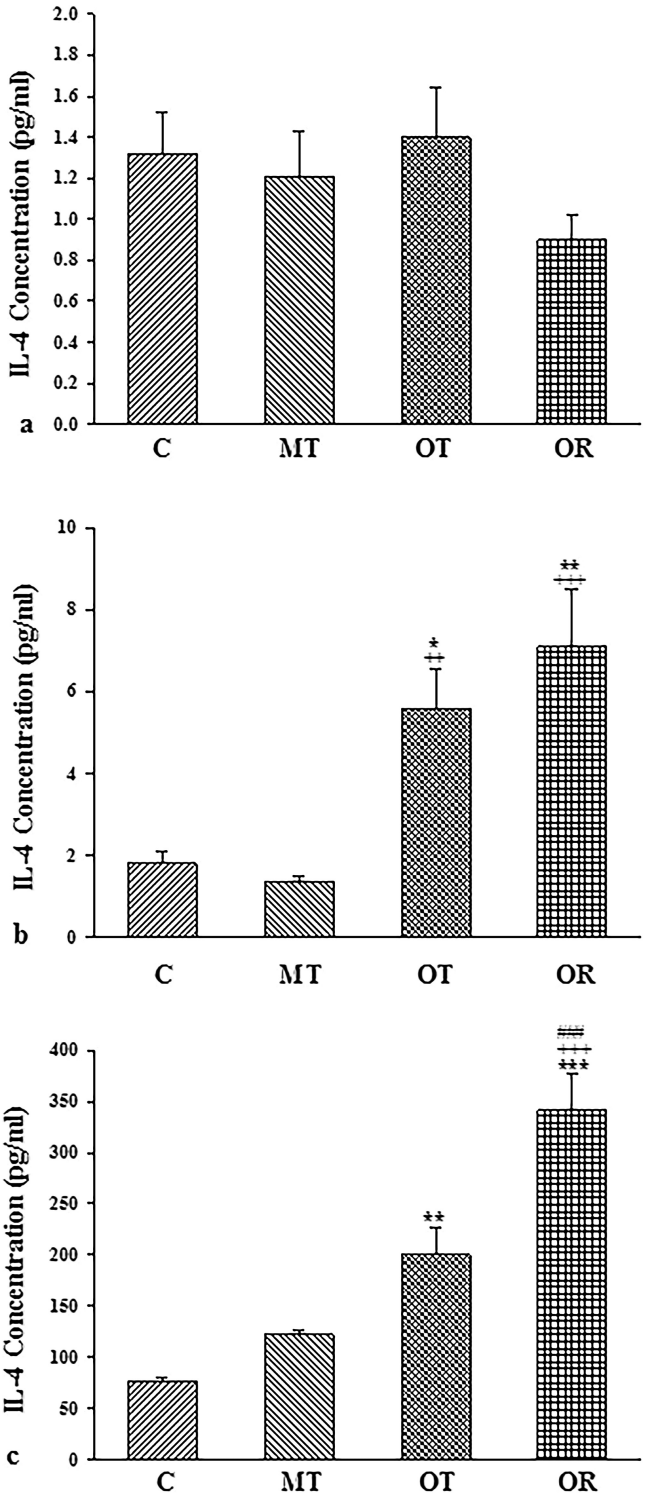

Fig. 3. IL-4 concentration of non-stimulated (a), PHA (b) and Con A (c)-stimulated splenocytes. The groups presented as sedentary control rats (C), moderately trained (MT), overtrained (OT), recovered after overtraining (OR), (for each group, $n = 6$). Data are mean ± SEM values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; compared to group C. $^{+}P < 0.05$, $^{+++}P < 0.001$; compared to group MT. $^{\#\#}P < 0.01$; compared to group OT.

Discussion

In the present study, a standard training protocol for overtraining was used. This protocol induced overtraining symptoms including lethargy, fatigue, and decline in performance, which remained even after two weeks of recovery in OR groups. Although different overtraining protocols (exercise speed (25–35 m/min), time (60–120 min), and slope (0–25) were used in previous studies, a decline in performance was commonly featured (da Rocha et al., 2017; Kadaja et al., 2010). Overtraining may cause increase in reactive oxygen species, depletion of blood glutamine, increment of gut permeability, skeletal tissue damage and stress hormone

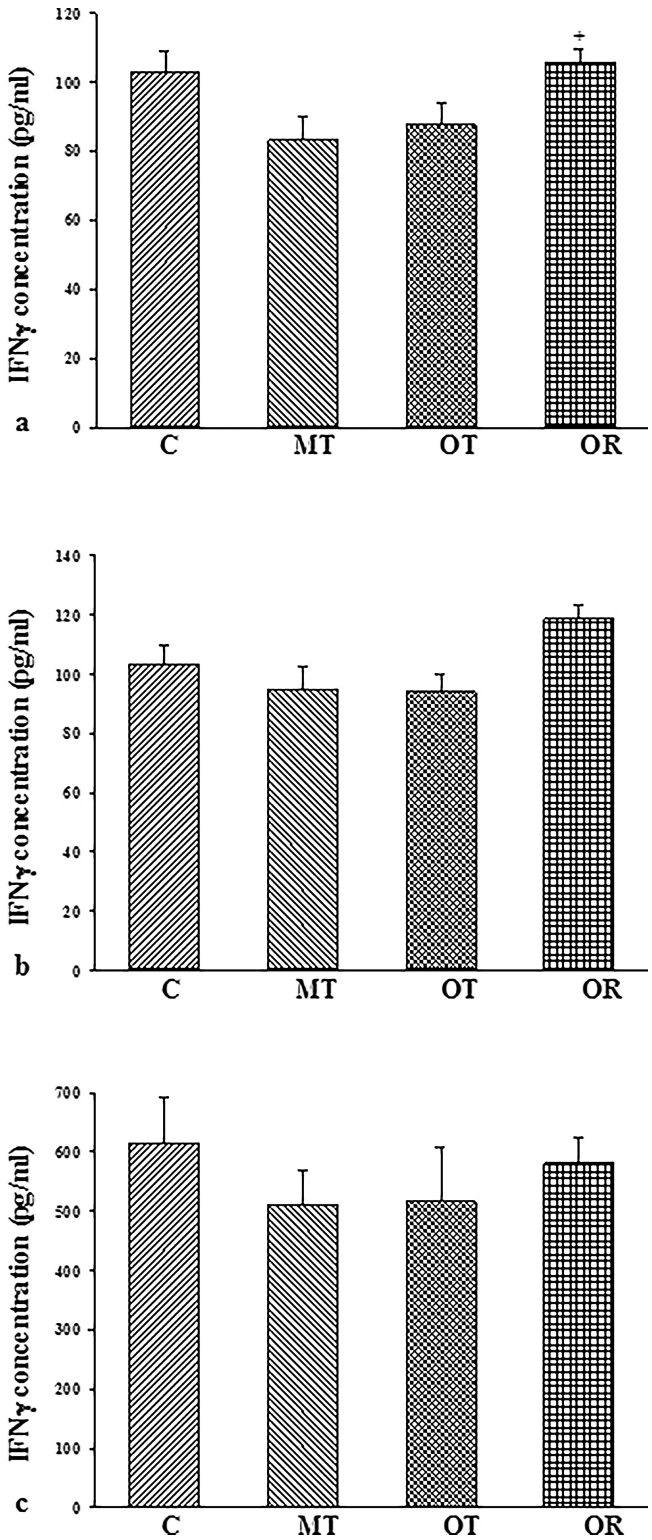

Fig. 4. IFNγ concentration of non-stimulated (a), PHA (b), and Con A (c)-stimulated splenocytes. The groups presented as sedentary control rats (C), moderately trained (MT), overtrained (OT), recovered after overtraining (OR), (for each group, n = 6). Data are mean ± SEM values. $^{+}P < 0.05$, compared to group MT.

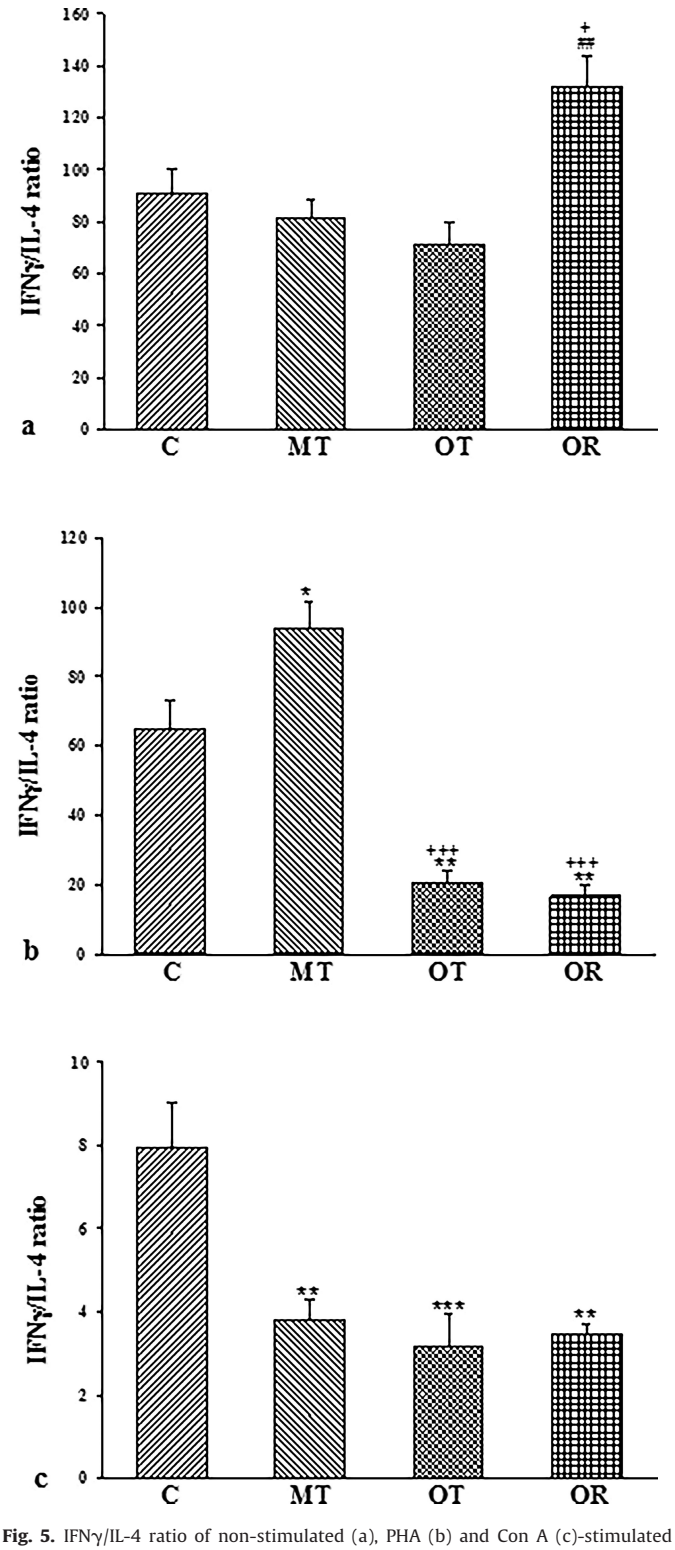

Fig. 5. IFNγ/IL-4 ratio of non-stimulated (a), PHA (b) and Con A (c)-stimulated splenocytes. The groups presented as sedentary control rats (C), moderately trained (MT), overtrained (OT), recovered after overtraining (OR), (for each group, n = 6). Data are mean ± SEM values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; compared to group C. $^{+}P < 0.05$, $^{+++}P < 0.001$; compared to group MT. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared to group OT.

elevation. These changes have been proposed as probable mechanisms underlying the imbalance in the immune system and elevation of pro-inflammatory and inflammatory cytokines after overtraining (da Silva and Macedo, 2011; Gleeson and Williams, 2013). This study showed that the level of training load may affect the isolated splenocytes viability, proliferation and Th1/Th2 cytokine secretion regardless of presence or absence of a mitogen. In addition, previous studies showed that splenocytes response to different mitogen varies in both sedentary and exercised animals (Gholamnezhad et al., 2015; Nielsen and

Pedersen, 1997). The results of the present study showed increased cell viability of isolated splenocytes in MT and OR groups in presence and absence of a mitogen as well as cell proliferation in OR group. IFNγ/IL-4 ratio was also increased in non-stimulated splenocytes of OR group, which may indicate correction of the immune system balance following a two-week recovery after overtraining by shifting the T helper cells activity toward Th2 pathway.

It was proposed that increasing of the training load would decrease the number of Th1 cells in circulation even in well-trained athletes, while the number of circulatory Th2 cells might not be affected. These changes may be due to alteration of pro/anti-inflammatory cytokine balance and elevations in circulating stress hormones (Walsh et al., 2011). It has been proposed that both epinephrine and cortisol may inhibit Th1 cytokine production, while IL-6 increases Th2 cytokine production. Lymphocyte redistribution and alteration of lymphoid and non-lymphoid tissues (the bone marrow, Peyer's patches and lung) homing may be another probable mechanism underlying overtraining-induced lymphopenia (Karsten et al., 2016). Th1/Th2 cytokine balance would affect autophagy/apoptosis in CD4 lymphocytes. IFNγ (a Th1 cytokine) up-regulated CD4 lymphocytes autophagy and depressed apoptosis, while IL4 (a Th2 cytokine) reversed those effects (Weng et al., 2013). In addition, increases of the ratio of IL-12 p40/p70 after exhaustive exercise, may be another mechanism for Th1 cellular immunosuppression (Suzuki et al., 2003).

Exercise increases plasma concentrations of stress hormones (glucocorticoids and catecholamines) as well as their receptor densities and affinities in the immune system cells (Leandro et al., 2006). In addition, overtraining induces hypoxic condition and excessive oxidative stress, as a situation similar to cardiopulmonary disease (Kontogianni et al., 2007; Weng et al., 2013). The level and type of stressor is a key factor, which may affect immune cells trafficking and apoptosis (Kruger et al., 2016). The alteration in cell trafficking is probably because of stress hormones inducing changes in cell integrin adhesion molecules and endothelial selectins. In addition, it was shown that increased levels of serum glucocorticoids enhance the sensitivity of β-adrenergic receptors and improve the response to catecholamines in immune cells (Sapolsky et al., 2000).

The mitogenic effect of Con A was more pronounced than PHA, which may be due to its specific effect on spleen lymphocytes. It was previously shown that PHA and Con A have more marked effects on T lymphocytes compared to B lymphocytes (Nielsen and Pedersen, 1997). In addition, the stimulatory effect of Con A on Th2 lymphocyte was more marked than its effect on Th1 lymphocyte, as it decreased IFNγ/IL4 ratio compared to non-stimulated cells (Gholamnezhad et al., 2015).

A previous study demonstrated that after severe exercise, the T cell proliferation decreases in response to PHA stimulation, while in Con A-stimulated cells, it was increased or remained unchanged. However, in recovery period, both responses were increased which confirm the result of this study (Nielsen and Pedersen, 1997). Leandro et al. (2006) showed that after moderate exercise, proliferation in Con A-stimulated T lymphocytes of thymus and mesenteric lymph nodes, decreased while splenocyte proliferation increased (Leandro et al., 2006). In another study, the effect of 1, 4, and 8 weeks of moderate treadmill exercise on rat peripheral blood and spleen lymphocyte subset fractions were evaluated. The result showed that short-term training decreased the CD8+ cell fraction in the blood and T cell fraction, the T/B lymphocyte ratio, and the CD4+/CD8+ ratio in the spleen compared to the control group. However, these parameters returned to those of the control group after 4 weeks, and authors concluded that at least 8 weeks of training was needed to observe the positive effect of moderate exercise on the immune system (Kim et al., 2003).

It was shown that regular exercise training reduced lymphocytes' apoptosis compared to sedentary subjects (Kruger and Mooren, 2014). Regular training may up-regulate cellular defence system like heat shock proteins thus increasing the cellular stress resistance (Fehrenbach et al., 2005). Unlike moderate training, it was shown that long-term exhaustive exercise not only reduces Th1 cells, but also reduces Th1 memory cells and shifts lymphocyte balance toward Th2 cells. It has been proposed that Th1 cells are more sensitive to glucocorticoids than Th2 and could be more markedly suppressed (Lancaster et al., 2005). In addition to inducing an elevated level of cortisol, overtraining may change the inflammatory cytokine balance which decreases B cell Ig synthesis and T cell proliferation (Gholamnezhad et al., 2014b; Kohut et al., 2004). Therefore, our findings showed that cumulative inhibitory effect of overtraining may cause a chronic suppression of immune system, which lasts for more than 2 weeks.

In the present study, the effect of physical activity was evaluated by performance test; however, VO2 peak was not measured, which is one of the limitations of this study. In addition, determination of T cell subtype percentage helps to better estimation of immune system changes after overtraining.

Conclusions

Two weeks recovery increased IFNγ/IL4 ratio compared to exercise group, only in non-stimulated splenocytes of over-trained animals. However, splenocyte response of exercise groups (MT, OT and OR) was shifted toward Th2 in the presence of mitogen. In addition, the results of the present study showed increased viability and proliferation of splenocytes in MT and specially OR groups, which indicated the beneficial effect of moderate exercise and recovery period after overtraining.

Conflict of interests

The authors have no conflict of interests to declare.

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References

Abbas, A.K., Lichtman, A.H., Pillai, S., 2012. Cellular and Molecular Immunology (Effector Mechanisms of Cell-Mediated Immunity). Elsevier/Saunders, Philadelphia.

Asea, A., Stein-Streilein, J., 1998. Signalling through NK1.1 triggers NK cells to die but induces NK T cells to produce interleukin-4. Immunology 93, 296–305.

Ceddia, M.A., Woods, J.A., 1999. Exercise suppresses macrophage antigen presentation. J. Appl. Physiol. 87, 2253–2258.

Cox, A.J., Pyne, D.B., Saunders, P.U., Callister, R., Gleeson, M., 2007. Cytokine responses to treadmill running in healthy and illness-prone athletes. Med. Sci. Sports. Exerc. 39, 1918–1926.

da Rocha, A.L., Pereira, B.C., Teixeira, G.R., Pinto, A.P., Frantz, F.G., Elias, L.L.K., et al., 2017. Treadmill slope modulates inflammation, fiber type composition, androgen, and glucocorticoid receptors in the skeletal muscle of overtrained mice. Front. Immunol. 8, 1378.

da Silva, F.O.C., Macedo, D.V., 2011. Physical exercise, inflammatory process and adaptive condition: an overview. Revista Brasileira de Cineantropometria and desempenho Humano 13, 320–328.

Dao, A.T., Zagaar, M.A., Levine, A.T., Salim, S., Eriksen, J.L., Alkadhi, K.A., 2013. Treadmill exercise prevents learning and memory impairment in Alzheimer's disease-like pathology. Curr. Alzheimer Res. 10, 507–515.

Dinarello, C.A., 1998. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. Int. Rev. Immunol. 16, 457–499.

Eyre, H., Baune, B.T., 2012. Neuroimmunological effects of physical exercise in depression. Brain Behav. Immun. 26, 251–266.

Fehrenbach, E., Niess, A.M., Voelker, K., Northoff, H., Mooren, F.C., 2005. Exercise intensity and duration affect blood soluble HSP72. Int. J. Sports. Med. 26, 552–557.

Gholamnezhad, Z., Boskabady, M.H., Hosseini, M., 2014a. Effect of *Nigella sativa* on immune response in treadmill exercised rat. BMC Complement. Altern. Med. 14, 437.
Gholamnezhad, Z., Boskabady, M.H., Hosseini, M., Sankian, M., Khajavi Rad, A., 2014b. Evaluation of immune response after moderate and overtraining exercise in wistar rat. Iran. J. Basic. Med. Sci. 17, 1–8.
Gholamnezhad, Z., Rafatpanah, H., Sadeghnia, H.R., Boskabady, M.H., 2015. Immunomodulatory and cytotoxic effects of *Nigella sativa* and thymoquinone on rat splenocytes. Food Chem. Toxicol. 86, 72–80.
Gleeson, M., Williams, C., 2013. Intense exercise training and immune function. Nestle Nutr. Inst. Workshop Ser. 76, 39–50.
Hohl, R., Ferraresso, R.L., De Oliveira, R.B., Lucco, R., Brenzikofer, R., De Macedo, D.V., 2009. Development and characterization of an overtraining animal model. Med. Sci. Sport. Exerc. 41, 1155–1163.
Jankowsky, J.L., Melnikova, T., Fadale, D.J., Xu, G.M., Slunt, H.H., Gonzales, V., et al., 2005. Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. J. Neurosci. 25, 5217–5224.
Kadaja, L., Eimre, M., Paju, K., Roosimaa, M., Podramagi, T., Kaasik, P., et al., 2010. Impaired oxidative phosphorylation in overtrained rat myocardium. Exp. Clin. Cardiol. 15, e116–127.
Kaiko, G.E., Horvat, J.C., Beagley, K.W., Hansbro, P.M., 2008. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 123, 326–338.
Karsten, K., Frank-Christoph, M., Christian, P., 2016. The immunomodulatory effects of physical activity. Curr. Pharm. Des. 22, 3730–3748.
Kim, H., Shin, M.S., Kim, S.S., Lim, B.V., Kim, H.B., Kim, Y.P., et al., 2003. Modulation of immune responses by treadmill exercise in Sprague-Dawley rats. J. Sport. Med. Phys. Fit. 43, 99–104.
Kohut, M.L., Arntson, B.A., Lee, W., Rozeboom, K., Yoon, K.J., Cunnick, J.E., et al., 2004. Moderate exercise improves antibody response to influenza immunization in older adults. Vaccine 22, 2298–2306.
Kontogianni, K., Messini-Nikolaki, N., Christou, K., Gourgoulianis, K., Tsilimigaki, S., Piperakis, S.M., 2007. DNA damage and repair capacity in lymphocytes from obstructive sleep apnea patients. Environ. Mol. Mutagen. 48, 722–727.
Kristensen, M., Albertsen, J., Rentsch, M., Juel, C., 2005. Lactate and force production in skeletal muscle. J. Physiol. 562, 521–526.
Kruger, K., Alack, K., Mink, L., Pfeifer, E., Gindler, K., Frech, T., et al., 2016. Apoptosis of T-cells subpopulations is differently affected by high-intensity interval exercise compared to isocaloric continuous exercise. Med. Sci. Sport. Exerc. 48, 817–818.
Kruger, K., Mooren, F.C., 2014. Exercise-induced leukocyte apoptosis. Exerc. Immunol. Rev. 20, 117–134.
Lancaster, G.I., Halson, S.L., Khan, Q., Drysdale, P., Wallace, F., Jeukendrup, A.E., et al., 2004. Effects of acute exhaustive exercise and chronic exercise training on type 1 and type 2 T-lymphocytes. Exerc. Immunol. Rev. 10, 91–106.
Lancaster, G.I., Khan, Q., Drysdale, P.T., Wallace, F., Jeukendrup, A.E., Drayson, M.T., et al., 2005. Effect of prolonged exercise and carbohydrate ingestion on type 1 and type 2 T lymphocyte distribution and intracellular cytokine production in humans. J. Appl. Physiol. 98, 565–571.
Leandro, C.G., Martins de Lima, T., Folador, A., Alba-Loreiro, T., do Nascimento, E., Manhães de Castro, R., et al., 2006. Physical training attenuates the stress-induced changes in rat T-lymphocyte function. Neuroimmunomodulation 13, 105–113.
Lira, F.S., Rosa, J.C., Pimentel, G.D., Tarini, V.A., Arida, R.M., Faloppa, F., et al., 2010. Inflammation and adipose tissue: effects of progressive load training in rats. Lipids Health Dis. 9, 109.
Meeusen, R., Duclos, M., Gleeson, M., Rietjens, G., Steinacker, J., Urhausen, A., 2006. Prevention, diagnosis and treatment of the overtraining syndrome. Eur. J. Sport Sci. 6, 1–14.
Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 2005. Two types of murine helper T-cell clone (I. Definition according to profiles of lymphokine activities and secreted proteins). J. Immunol. 175, 5–14.
Nielsen, H.B., Pedersen, B.K., 1997. Lymphocyte proliferation in response to exercise. Eur. J. Appl. Physiol. Occup. Physiol. 75, 375–379.
Ogonovszky, H., Sasvári, M., Dosek, A., Berkes, I., Kaneko, T., Tahara, S., et al., 2005. The effects of moderate, strenuous, and overtraining on oxidative stress markers and DNA repair in rat liver. Can. J. Appl. Physiol. 30, 186–195.
Ortega, E., Giraldo, E., Hinchado, M.D., Martin, L., Garcia, J.J., De la Fuente, M., 2007. Neuroimmunomodulation during exercise: role of catecholamines as 'stress mediator' and/or 'danger signal' for the innate immune response. Neuroimmunomodulation 14, 206–212.
Peake, J.M., Neubauer, O., Walsh, N.P., Simpson, R.J., 2017. Recovery of the immune system after exercise. J. Appl. Physiol. 122, 1077–1087.
Perrin, P.J., Davis, T.A., Smoot, D.S., Abe, R., June, C.H., Lee, K.P., 1997. Mitogenic stimulation of T-cells reveals differing contributions for B7-1 (CD80) and B7-2 (CD86) costimulation. Immunology 90, 534–542.
Petersen, A.M., Pedersen, B.K., 2005. The anti-inflammatory effect of exercise. J. Appl. Physiol. 98, 1154–1162.
Pimlott, N., 2010. The miracle drug. Can. Fam. Physician. 56, 407–409.
Radak, Z., Chung, H.Y., Koltai, E., Taylor, A.W., Goto, S., 2008. Exercise, oxidative stress and hormesis. Ageing. Res. Rev. 7, 34–42.
Ru, W., Peijie, C., 2009. Modulation of NKT cells and Th1/Th2 imbalance after alpha-GalCer treatment in progressive load-trained rats. Int. J. Biol. Sci. 5, 338–343.
Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.
Seder, R.A., Paul, W.E., 1994. Acquisition of lymphokine-producing phenotype by CD4+ T-cells. Annu. Rev. Immunol. 12, 635–673.
Silva, L.C., de Araujo, A.L., Fernandes, J.R., Matias Mde, S., Silva, P.R., Duarte, A.J., et al., 2016. Moderate and intense exercise lifestyles attenuate the effects of aging on telomere length and the survival and composition of T-cell subpopulations. Age (Dordr) 38, 24.
Suzuki, K., Nakaji, S., Kurakake, S., Totsuka, M., Sato, K., Kuriyama, T., et al., 2003. Exhaustive exercise and type-1/type-2 cytokine balance with special focus on interleukin-12 p40/p70. Exerc. Immunol. Rev. 9, 48–57.
Wallberg, L., Mikael Mattsson, C., Enqvist, J.K., Ekblom, B., 2011. Plasma IL-6 concentration during ultra-endurance exercise. Eur. J. Appl. Physiol. 111, 1081–1088.
Walsh, N.P., Gleeson, M., Shephard, R.J., Woods, J.A., Bishop, N.C., Fleshner, M., et al., 2011. Position statement. Part one: immune function and exercise. Exerc. Immunol. Rev. 17, 6–63.
Weng, T.P., Huang, S.C., Chuang, Y.F., Wang, J.S., 2013. Effects of interval and continuous exercise training on CD4 lymphocyte apoptotic and autophagic responses to hypoxic stress in sedentary men. PLoS One 8, e80248.
Zhao, G., Zhou, S., Davie, A., Su, Q., 2012. Effects of moderate and high intensity exercise on T1/T2 balance. Exerc. Immunol. Rev. 18, 98–114.