

Overfrequent training does not induce oxidative stress and inflammation in blood and heart of rats

Short title: Oxidative stress and inflammation in frequent exercise

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Summary

The aim of our research was to evaluate the changes in levels of cytokines and redox state parameters in blood and isolated heart of rats subjected to different swimming protocols. Rats were divided into 3 groups: 1) controls, 2) moderately trained rats that during all 12 weeks swam 1 h/day, 5 days/week, and 3) overfrequently trained rats that in 10th week swam twice, 11th week 3 times, and in 12th week 4 times a day for 1 hour. After sacrificing, blood from jugular vein was collected, and the heart excised and perfused on a Langendorff apparatus. Samples of the coronary effluent were collected during coronary autoregulation. Levels of superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO) and thiobarbituric acid reactive substances (TBARS) were measured in plasma and coronary effluent, while reduced glutathione (GSH), activities of superoxide dismutase (SOD) and catalase (CAT) were measured in erythrocytes. Venous blood was also used for interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α) determination. Moderate training protocol induced the decrease of TBARS in plasma, while both training protocols induced the decrease of O_2^- and H_2O_2 in coronary effluent. There was no significant difference in levels of cytokines between groups. The results of study add evidence about beneficial effects of moderate-intensity training on blood and cardiac redox and state of rats, and furthermore, shows that exercising frequently, if the intensity stays within moderate range, may not have detrimental effects.

Keywords: overtraining, redox state, cytokines, swimming, isolated rat heart, frequent training

Introduction

According to the contemporary scientific data, in a well-planned and programmed training process, the training load increases stepwise and the periods of rest between the exercise bouts are sufficiently long to ensure regeneration of the muscle functions, but still short enough to not allow the regression of supercompensation (Seene *et al.* 2004). Every athletic training program includes a component of repetitive overloading, but with an inadequate recovery time, such overloading may produce undesired effects, such as chronic fatigue and the absence of performance improvement (Kadaja *et al.* 2010), i.e. the overtraining syndrome (OTS). Since it is not ethical to train athletes in such a manner that they develop OTS, in order to explore the etiology and symptoms of this phenomenon, animal overtraining models have been developed (Hohl *et al.* 2009, Pereira *et al.* 2012). Animal (rat) models often allow for more invasive, extensive, and homogenous experimental designs than human models. These rat-based exercise studies may provide more information and help to elucidate the effects of different training regimes on numerous body structures and functions.

The aim of our research was to evaluate the changes in levels of cytokines and redox state parameters in blood and isolated heart of rats subjected to different swimming protocols during 12 weeks. We hypothesized that moderate intensity swimming training (60 minutes of daily swimming, 5 times a week), which is the most commonly used rat swimming protocol in exercise physiology studies, would not induce significant rise of inflammatory markers in rats' blood, but would induce upregulation of rats' endogenous antioxidative defence. On the other side, we hypothesized that increasing frequency of trainings and decreasing recovery time between trainings may lead towards the rise of levels of inflammatory markers, as well as undesirable systemic and cardiac redox state changes. We based our assumptions on results of

previously published studies that used similar rat exercise protocols (Ogonovszky *et al.* 2005, Zoppi and Macedo, 2008, Lira *et al.* 2010, Dong *et al.* 2011, Ferraresso *et al.* 2012, Gholamnezhad *et al.* 2013,), and showed existence of relationship between OTS, oxidative stress and inflammation in different tissues.

Materials and methods

Ethical approval

The study was performed in the cardiovascular laboratory of the Faculty of Medical Sciences, University of Kragujevac, Serbia. It was approved by Ethical committee of the Faculty, and performed according to the Faculty's rules for the welfare of laboratory animals, which are in consent with Good laboratory practice and European Council Directive (86/609/EEC).

Subjects

Twenty one Wistar albino rat (females, eight weeks old, body weight 200 ± 50 g) was subjected to the study's protocol. The sample size was calculated based on previously published study (Ferraresso *et al.* 2012), using an appropriate computer program (Faul *et al.* 2007). Rats were housed in cages (3 rats per cage) in a room where temperature was maintained on 25°C , with 12h of light daily. They consumed commercial rat food (20% protein rat food, Veterinary institute Subotica, Serbia) and water *ad libitum*.

Exercise training protocol

Rats were divided into three groups: control (C), moderately trained (MT) and overfrequently trained (OT) group) and subjected to 12 weeks of swimming training according to

different training protocols. Overfrequently trained rats swam according to the protocol in Table 1, which represents previously developed overtraining animal model (Hohl *et al.* 2009), performed in water instead of treadmill (Stojanovic Tomic *et al.* 2015). It is based on increasing frequency of trainings, decreasing recovery time between trainings, and increasing total volume of training. Rats chosen for the moderately trained group trained according to the protocol in Table 1 until the end of 9th week. Then, they continued to swim once a day for 60 minutes until the end of 12th week. Rats from the control group were put in water for 3 minutes a day, 5 days a week, all 12 weeks, in order to achieve the water-induced stress effect. Rats swam in a specially constructed swimming pool made of glass (80x60x100cm) in which water temperature (34C°) was maintained by an electric heater, and a pump continuously made waves in order to prevent rats from floating. The swimming was continuously supervised.

Physical capacity test

During 12 weeks of exercise training program, rats from all 3 groups were subjected to 6 endurance swimming tests (schedule of testing is presented in Table 1). Physical capacity tests were performed in order to assess if rats experienced OTS, since the only certain sign of this syndrome is a decrease in performance during competition or training (Meeusen *et al.* 2013). Every physical capacity test was performed 30-32 hours after the last training session, and 30-32 hours before the next training session. The test was performed with a weight equivalent to 10% of body weight, which was attached around the waist of each rat using a rubber band (Bocalini *et al.* 2010). Rats from all groups were individually observed to determine swimming time until exhaustion. Exhaustion was defined as the point when the rat could not swim up to the water surface for 10s.

Samples preparation and biochemical analyses

Thirty six hours after the last physical capacity test, after a short-term ether narcosis and premedication with heparin as an anticoagulant, rats were sacrificed by decapitation. Blood for analysis of systemic oxidative stress and inflammation was collected from the jugular vein into Vacutainer test tube containing sodium citrate anticoagulant. Blood was centrifuged to separate plasma and red blood cells (RBCs). Blood samples were analyzed immediately. Following parameters of oxidative stress were measured in these samples: superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), nitrites (NO_2^-) as measure of nitric oxide (NO), thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) activity. Levels of cytokines, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α), were also measured in those samples. Biochemical parameters were measured spectrophotometrically (Specord S-600 Analytik Jena).

Immediately after venous blood sampling, after emergency thoracotomy and rapid cardiac arrest by superfusion with ice-cold isotonic saline, the heart was excised, the aorta cannulated and the heart retrogradely perfused on a Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary). The composition of the non-recirculating Krebs-Henseleit perfusate was as follows (mmol/l): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O_2 plus 5% CO_2 and warmed to 37°C (pH 7.4). Coronary perfusion pressure (CPP) was gradually increased (from 40 to 120 mmHg) in order to establish coronary autoregulation. During each CPP coronary venous effluent was collected for the purpose of determination of levels of prooxidants (O_2 , H_2O_2 , NO_2 ,

TBARS). These prooxidant analyses were performed using the same methods as when analysing plasma samples.

Superoxide anion radical determination

The level of O_2^- was measured using NBT (nitro blue tetrazolium) reaction in TRIS-buffer combined with plasma samples and read at 530 nm (Auclair and Voisin 1985).

Hydrogen peroxide determination

The protocol for measurement of H_2O_2 is based on oxidation of phenol red in the presence of horseradish peroxidase (Pick and Keisari 1980). 200 μ l of plasma with 800 μ l PRS (Phenol Red Solution) and 10 μ l POD (Horseradish Peroxidase) were combined (1:20). The level of H_2O_2 was measured at 610 nm.

Nitric oxide determination

NO decomposes rapidly to form stable metabolite nitrite/nitrate products. NO_2^- was determined as an index of nitric oxide production with Griess reagent (Green *et al.* 1982). 0.1 ml 3N PCA (perchloride acid), 0.4 ml 20 mM EDTA (ethylenediaminetetraacetic acid) and 0.2 ml plasma were put on ice for 15 min, then centrifuged 15 min at 6000 rpm. After pouring off the supernatant, 220 μ l K_2CO_3 was added. NO_2^- were measured at 550 nm. Distilled water was used as a blank probe.

Thiobarbituric acid reactive substances

The degree of lipid peroxidation in plasma was estimated by measuring TBARS using 1% TBA (thiobarbituric acid) in 0.05 NaOH, incubated with plasma at 100°C for 15 min and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml TCA (trichloro acetic acid), then samples were put on ice for 10 minutes, and centrifuged for 15 min at 6000 rpm. This method was described previously (Ohkawa *et al.* 1979).

Determination of antioxidant enzymes

Isolated RBCs were washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl and hemolysates containing about 50 g Hb/l (McCord and Fridovich 1969), were used for the determination of CAT activity. CAT activity was determined according to Beutler (1982). Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin (Tsuchihashi 1923). Then 50 µl CAT buffer, 100 µl sample and 1 ml 10 mM H₂O₂ were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich (1972). A hundred µl lysate and 1ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470nm.

Determination of glutathion

The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5,5- dithio-bis-6,2-nitrobenzoic acid, using method by Beutler (1975).

Determination of interleukin 6 and tumor necrosis factor alpha

The serum levels of IL-6 and TNF- α were measured with a highly sensitive enzymatic immunosorbent assay (ELISA) by rat interleukin kits (R&D Systems, USA). Before use, the standards were dissolved in phosphate buffered saline (pH 7.2), so that the initial concentrations were 2000 pg/ml. The created stocks were serially 7 times twofold diluted in reagent diluents, in order to get a standard curve with 7 points. 100 μ l of work concentration of a capture antibody was poured into the wells of polystyrene microtiter plate (MTP). The plates were sealed, incubated and left overnight at room temperature, after which they were wash buffered. All the samples were dissolved in solvent, in a 1:4 ratio. The dissolved samples and prepared standards were poured into MTP, covered with adhesive foil and left for 2 hours at room temperature. After incubation and rinsing, the well, following an adding of 100 μ l of work concentration of a detection antibody, were once more covered by adhesive foil and left for another 2 hours at room temperature. After a new incubation and rinsing, Streptavidin-HRP (Streptavidin horseradish peroxidase) was added (100 μ l). The incubation was interrupted after 20 minutes at room temperature, without being exposed to direct sunlight. Following that, 100 μ l of substrate solution was poured in as well as 50 μ l of stop solution (2N H₂SO₄). The optical density was measured by a microplate reader (Zenyth, Anthos, UK) set at 450 nm (Crowther 1995).

Statistics

The statistical analysis was performed using SPSS 19.0 for Windows. Results in tables and in figures are expressed as means \pm standard error of the mean. Due to low number of cases, nonparametric tests were used. For difference between unrelated samples Kruskal Wallis and Mann Whitney tests were used, and for difference between related samples Friedman and Wilcoxon test was used. Alpha level for significance was set to $P < 0.05$.

Results

Although rats were subjected to low, moderate and strenuous swimming training process during 12 weeks, C, MT and OT group did not differ significantly in parameters of cardiac hypertrophy (Table 2), or physical capacity (Table 3). The result on physical capacity test significantly improved in all rats when they were subjected to it for the second time (T1 vs T2: $P=0.046$ in C group, $P=0.028$ in MT group, $P=0.043$ in OT group), but no significant difference between any other two successive measurements was observed.

The analysis of venous blood samples of sacrificed rats (Table 4) showed that the only significant difference between groups was in levels of TBARS, whose levels were significantly lower in MT group compared with the C group ($P=0.006$). Levels of other pro/antioxidants (O_2^- , H_2O_2 , NO, SOD, CAT, GSH) were not found to be significantly different between groups. There was no statistically significant difference between groups neither in IL-6 nor in TNF- α level.

Analysis of prooxidant parameters in the isolated rat heart effluent (Figures 1A, 1B, 1C, 1D) during coronary autoregulation showed that there was no statistically significant difference in levels of any measured parameter between MT and OT groups, while when those groups were compared to C group, C group had significantly higher levels of O_2^- and H_2O_2 during periods of specific coronary perfusion pressures.

In case of O_2^- levels in coronary effluent, following differences were found: 1) C vs MT group: $P=0.028$ during CPP 40mmHg; $P=0.016$ during CPP 60mmHg; $P=0.028$ during CPP 120mmHg, 2) C vs OT group: $P=0.018$ during CPP 60mmHg; $P=0.011$ during CPP 80mmHg.

Levels of H₂O₂ in coronary effluent were found to be significantly different between groups (higher in C group) during following CPP values: 1) C vs MT group: P=0.006 during CPP 100mmHg; P=0.004 during CPP 120mmHg, 2) C vs OT group: P=0.028 during CPP 100mmHg.

Discussion

To maximize physical performance, athletes and coaches manipulate training load through adjustments in duration, frequency, and intensity or through a reduction of the regenerative period (Ferraresso *et al.* 2012). However, if the exercise session lasts too long and training sessions are so frequent that they interrupt the recovery phase, the necessary adaptation does not occur (Lehmann *et al.* 1999). Thus, the aim of our research was to explore the effects of overfrequent swimming on parameters of oxidative stress and inflammation in blood and isolated heart of rats.

The first aim of the study was to assess the effects of overfrequent swimming on redox state of rats on both systemic and local level. When analyzing systemic oxidative stress, we measured levels of redox state parameters in venous blood of rats (O₂⁻, H₂O₂, NO₂⁻ and TBARS in plasma; GSH levels, SOD and CAT activity in RBCs). The only statistically significant difference between groups that swam according to different training protocols was found in plasma levels of TBARS, which were significantly lower in moderately trained than in the control rats. Measuring TBARS is one of the ways to assess the degree of lipid peroxidation, which is one of the adverse redox change consequences, and their lower levels certainly mean better redox state. Unfortunately, there are no studies that used training protocol similar to ours that explored redox parameters in blood, except that Dong and colleagues measured

malondialdehyde (MDA) levels in overtrained rats blood, and found it significantly increased (Dongl *et al.* 2011). However, our overfrequently trained rats experienced no oxidative stress. As the results of physical capacity tests in our study show, the applied overfrequent swimming training protocol did not induce OTS in rats, so this can explain the absence of adverse redox state in our overfrequently trained rats. Interestingly, although the differences were not shown to be statistically significant, the overfrequently trained rats had the highest levels of all three measured endogenous antioxidants, which is probably the adaptation that occurred in order to cope with increased oxidative demands of multiple daily training sessions. In addition to the data on redox state in blood, as well as on the data on oxidative stress in coronary effluent which will be discussed later, and on recently published data on the cardiodynamics of hearts isolated from rats used in this project (Stojanovic Tomic *et al.* 2015), it seems that the performed overfrequent protocol induced desirable changes in both function of the heart and redox state of rats to the equal or even to the greater extent than the moderate, usually performed swimming training regimen. This gives guidelines for future swimming overtraining protocol planning, such as that intensity and maybe volume of a single session, rather than only frequency, must be increased. This hypothesis may be supported by the results of Burneiko and colleagues (Burneiko *et al.* 2004) who showed that, in contrast to the loadless training program, the training program that included swimming with 2% of rat's body weight was deleterious, increasing both myocardial and plasma oxidative stress.

Endurance exercise training is known to promote beneficial adaptations to numerous tissues including the heart. Many studies have explored the impact of exercise training on myocardial antioxidant capacity, and although there is some inconsistency in results of those studies, there is emerging evidence to suggest that exercise increases antioxidant enzymes in the

heart (Powers et al. 2014). The difference between numerous other studies and our study lies in the site where redox parameters were measured. Most studies measured levels of redox state parameters in of the whole heart tissue, while we measured levels of prooxidants (O_2^- , H_2O_2 , NO_2^- and TBARS) in the coronary effluent (we did not measure levels of antioxidants since methods for their estimation require RBCs). Oxidative stress detected this way represent oxidative stress in the endocardium of the left ventricle, although it may also originate from the endothelium of the coronary circulation. Our analysis of prooxidant parameters in the coronary venous effluent during coronary autoregulation on the Langendorf apparatus showed that, compared to the controls, hearts from both moderately trained and overfrequently trained rats produced lower levels of O_2^- and H_2O_2 . This is important since it was shown that O_2^- and H_2O_2 induce apoptosis (Chabi *et al.* 2008) by opening the permeability transition pore and triggering the release of proapoptotic proteins into the cytosol (Jackson *et al.* 2002). The results of our study cannot directly be compared to other studies that explored the effects of exercise on the myocardium redox state due to different methodology, but it may be said that our results support the hypothesis that, in terms of redox state, moderate exercise exhibits protective effects on the heart muscle.

Levels of cytokines measured in blood of rats subjected to our study protocol did not significantly differ. Average value of IL-6 was somewhat lower in trained rats than in controls, while levels of TNF- α were somewhat increased in trained rats, but the differences were not found to be statistically significant. Thus, our results suggest that moderate intensity training does not induce inflammation in blood of rats. This is in contrast to the study performed by Gholamnezhad and colleagues (2013), who applied training protocol similar to ours and explored levels of cytokines in blood of rats, both in rest and after acute exercise. They found increased

IL-6 and TNF- α levels in overtrained rats (compared to controls), both immediately after acute exercise and 24 hours later, and thus concluded that inadequately dosed physical activity induces silent inflammation. Lira and colleagues (2010) also reported the increase of inflammatory cytokines in overtrained rats, but in white adipose tissue.

Finally, the results of our study show that applied overfrequent swimming protocol, based on previously published running overtraining protocol, was not successful in inducing OTS in rats. This may be the consequence of differences between swimming and running training in rats (Kregel *et al.* 2006; Seo *et al.* 2014), or sex differences, since all the studies we compared our results to, used male rats. In most animal studies, females and males differ in the extent of the response to an exercise; thus, the outcomes of a given exercise intervention cannot be generalized for both sexes (Foryst-Ludwig and Kintscher 2013).

Conclusion

The results of our study add evidence about beneficial effects of regular moderate exercise on blood and cardiac redox state of rats, and furthermore, shows that exercising frequently, if the intensity stays within moderate range, may not have detrimental effects, but on the contrary. Further research is needed to discover the elements of the training load that would induce overtraining in rats when using swimming as the chosen physical activity, and thereafter explore the relationship between structure, function and biochemistry of the heart and vascular system in this unexplained phenomenon.

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TABLES

TABLE 1: Overfrequent swimming protocol (AW: adaptation to water; AT1 and AT2: adaptive training phases 1 and 2; T2x, T3x, and T4x: overfrequent training phase in which daily training frequency is increased to two, three, and four times).

Week No.	Training phase	Swimming duration	Daily number of training sessions	Recovery between training sessions	Physical capacity test
1	AW	5-15min	1	24h	T1
2	AT1	20min	1	24h	/
3	AT1	30min	1	24h	/
4	AT1	45min	1	24h	/
5	AT1	60min	1	24h	T2
6	AT2	60min	1	24h	/
7	AT2	60min	1	24h	/
8	AT2	60min	1	24h	/
9	AT2	60min	1	24h	T3
10	T2x	60min	2	4h	T4
11	T3x	60min	3	3h	T5
12	T4x	60min	4	2h	T6

TABLE 2: Body and heart weight ($X \pm SE$) of rats (C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).

Parameter	C group (n=7)	MT group (n=7)	OT group (n=7)	Statistics
Body weight (g)	368.33±14.70	361.66±14.24	363.33±9.18	P>0.05
Heart weight (mg)	1566.66±33.33	1616.66±65.40	1618.33±116.66	P>0.05
Heart/body weight (mg/g)	4.27±0.14	4.48±0.15	4.43±0.25	P>0.05

TABLE 3: Results ($X \pm SE$) of the physical capacity tests (C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).

Test No.	C group (n=7)	MT group (n=7)	OT group (n=7)	Two independent samples test
T1 (sec)	79.66±12.65	83.16±7.80	87.83±13.26	P>0.05
T2 (sec)	141.66±17.35	159.50±21.81	160.83±22.24	P>0.05
T3 (sec)	127.33±18.13	185.00±12.13	170.50±17.02	P>0.05
T4 (sec)	172.50±13.40	210.66±33.36	220.50±31.14	P>0.05
T5 (sec)	132.33±15.45	200.00±29.95	214.16±31.11	P>0.05
T6 (sec)	155.83±21.92	195.50±19.49	185.50±27.35	P>0.05
Two related samples test	T1 vs T2: P<0.05	T1 vs T2: P<0.05	T1 vs T2: P<0.05	

TABLE 4: Parameters ($X \pm SE$) of redox state and inflammation in blood of rats (C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).

Parameter	C group (n=7)	MT group (n=7)	OT group (n=7)	Two independent samples test
O_2^- (nmol/ml)	9.88 \pm 2.78	14.11 \pm 3.28	12.08 \pm 2.7	P>0.05
H_2O_2 (nmol/ml)	1.36 \pm 0.39	1.42 \pm 0.47	1.23 \pm 0.23	P>0.05
NO_2^- (nmol/ml)	2.67 \pm 0.46	1.9 \pm 0.19	2.13 \pm 0.44	P>0.05
TBARS (μ mol/ml)	0.60 \pm 0.02	0.16 \pm 0.04	0.39 \pm 0.10	C vs MT: P<0.01
SOD (U/g Hb x 10^3)	24.42 \pm 8.66	36.63 \pm 14.82	56.98 \pm 15.30	P>0.05
CAT (U/g Hb x 10^3)	7.70 \pm 1.28	8.12 \pm 1.65	9.41 \pm 1.88	P>0.05
GSH (nmol/ml RBCs)	7816.90 \pm 958.80	7193.35 \pm 2839.92	8756.74 \pm 1549.23	P>0.05
IL-6 (pg/ml)	152.05 \pm 6.46	135.28 \pm 5.48	136.28 \pm 7.99	P>0.05
TNF- α (pg/ml)	46.84 \pm 10.74	93.16 \pm 20.28	79.83 \pm 17.60	P>0.05

FIGURES

Fig 1A. Values of superoxide anion radical ($X \pm SE$ nmol/ml) in effluent from an isolated rat heart during different coronary perfusion pressures (a – significant difference between controls (n=7) and moderately trained (n=7) rats, b – significant difference between controls (n=7) and overfrequently trained (n=7) rats, C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).

Fig 1B. Values of hydrogen peroxide ($X \pm SE$ nmol/ml) in effluent from an isolated rat heart during different coronary perfusion pressures (a – significant difference between controls (n=7) and moderately trained (n=7) rats, b – significant difference between controls (n=7) and overfrequently trained (n=7) rats, C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).

Fig 1C. Values of nitrites ($X \pm SE$ nmol/ml) in effluent from an isolated rat heart during different coronary perfusion pressures (C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).

Fig 1D. Values of index of lipid peroxidation ($X \pm SE$ μ mol/ml) in effluent from an isolated rat heart during different coronary perfusion pressures (C group – control group, MT group – moderately trained group, OT group – overfrequently trained group).







