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An Experimental Study on Investigation of Myokine Responses to Acute and Chronic Swimming Exercise in Mice

Farelerde Akut ve Kronik Yüzme Egzersizine Miyokin Cevaplarının İncelenmesi Üzerine Deneysel Bir Çalışma

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This study was prepared based on the findings of Egem Burcu ÜNAL's medical doctor thesis study titled "Investigation of myokine responses to acute and chronic swimming exercise in mice" (Denizli: Pamukkale University; 2020).

ABSTRACT Objective: This study aimed to investigate the changes in the plasma levels of ciliary neurotrophic factor (CNTF), CXC ligand 1 (CXCL-1), cardiotrophin 1 (CT-1), oncostatin M (OSM), myostatin (MSTN) myokines in response to acute-chronic swimming exercise in mice. **Material and Methods:** 70 mice were divided into control, acute swimming (30 min) and chronic swimming (30 min/day, 5 days/week, 6 weeks) groups. Exercising mice were further divided into 3 in terms of the time passed (3, 24, 48 h) following the last exercise session. Plasma CNTF, CXCL-1, CT-1, OSM and MSTN levels were determined using commercial kits. **Results:** No statistically significant time-dependent alteration in plasma levels of CNTF, CXCL-1, CT-1, OSM, MSTN was determined. **Conclusion:** The fact that there is no change in plasma myokine concentrations at 3, 24 and 48 h following exercises may not exclude the possibility that these myokines play role in exercise-induced adaptations. If different sample collection times had been chosen, possible changes in these myokines may have been detected.

Keywords: Adaptation; myokine; swimming

ÖZET Amaç: Bu çalışmada, farelerde akut-kronik yüzme egzersizine yanıt olarak siliyer nörotrofik faktör [ciliary neurotrophic factor (CNTF)], CXC ligand 1 (CXCL-1), kardiyotrofin 1 [cardiotrophin 1 (CT-1)], onkostatin M (OSM), miyostatin (MSTN) miyokinlerinin plazma düzeylerindeki değişimlerin araştırılması amaçlandı. **Gereç ve Yöntemler:** 70 fare; kontrol, akut yüzme (30 dk) ve kronik yüzme (30 dk/gün, 5 gün/hafta, 6 hafta) gruplarına ayrıldı. Egzersiz yapan fareler, son egzersiz seansından sonra geçen süreye göre (3, 24, 48 saat) ayrıca 3'e bölündü. Plazma CNTF, CXCL-1, CT-1, OSM ve MSTN düzeyleri ticari kitler kullanılarak ölçüldü. **Bulgular:** CNTF, CXCL-1, CT-1, OSM, MSTN'nin plazma düzeylerinde zamana bağlı istatistiksel olarak anlamlı bir değişiklik saptanmadı. **Sonuç:** Egzersiz sonrası 3, 24 ve 48. saatlerde plazma miyokin konsantrasyonlarında herhangi bir değişiklik olmaması, bu miyokinlerin egzersize bağlı adaptasyonlarda rol oynama olasılığını dışlamayabilir. Farklı numune toplama zamanları seçilseydi bu miyokinlerdeki olası değişiklikler tespit edilebilirdi.

Anahtar Kelimeler: Adaptasyon; miyokin; yüzme

In recent years, with the discovery of muscle-released proteins called myokines and revealing the physiological roles of these substances have become the focus of attention in scientific community.[1] It is known that exercise has many beneficial effects on the organism; especially the musculoskeletal system; some of which are mediated by myokines.[2] Myokines released by skeletal muscle in response to exercise regulate muscle growth and lipid metabolism with their autocrine and paracrine effects.[3] Thus, the muscle is adapted to exercise.[4]

There are studies examining serum and various tissue levels of myokines. In a study conducted on mice, serum CXC ligand 1 (CXCL-1) levels in the

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control group were measured as 52±1.9 pg/mL.[5] Two hours after performed 1 h of swimming exercise, serum CXCL-1 increased significantly compared to control levels (127±6.3 pg/mL).[5] In the same study, CXCL-1 mRNA expression increased 41-fold in the liver and 2.4-fold in the gastrocnemius muscle after a single exercise session.[5] Although the role of ciliary neurotrophic factor (CNTF) in the nervous system has a wide area of research, there are no studies examining its serum or plasma levels since it has recently attracted attention as a myokine. In a study where serum cardiotrophin 1 (CT-1) values of control group rats were measured as 232.9±20.9 pg/mL, when the effects of running and swimming exercises were compared, they were measured as 350.5±38.0 and 331.2±24.9 pg/mL, respectively.[6] In another study, since serum oncostatin M (OSM) levels were very low in the control group of mice, they could only be detected in 4 out of eight mice.[7] After 1 h of swimming, OSM increased significantly and was detectable (6.9±1.2 pg/mL) in all mice.[7] Two hours after exercise, serum OSM returned to control levels.[7] Following swimming exercise, OSM expression was upregulated only in the gastronemius muscle, whereas OSM expression was unchanged in other tissues (spleen, liver, and adipose tissue).[7] There is no study reporting normal and exercise-related serum/plasma myostatin (MSTN) levels in mice. In a study examining the muscle tissues of rats, two sessions of swimming did not change MSTN expression, while 4 weeks of swimming significantly reduced MSTN mRNA expression.[8] In addition, MSTN mRNA content in cardiac muscle was lower than that in skeletal muscle and was significantly increased by swimming exercise.[8]

Studies have examined alterations in the levels of some myokines in response to various exercise protocols. It has been shown that CXCL-1 plays a role in neovascularization, inflammation, and wound healing processes and has corrective effects on diet-induced obesity.[5,9] CNTF, CT-1 and OSM, members of the myokine family called "leukemia inhibitory factor receptor (LIFR) myokines", attract attention due to their close relationship with exercise.[10] LIFR myokines play role in muscle growth/differentiation, muscle response to exercise, neurostimulation of muscle, and recruitment of inflammatory cells to injured muscle areas.[3] Our knowledge is quite limited due to the recent discovery of these myokines and the lack of sufficient studies to reveal their relationship with exercise. It has been shown that muscle mass can be preserved by CNTF replacement.[11] CT-1 is affected by skeletal muscle activity and its levels change with exercise.[4,6] CNTF, CT-1 and OSM are likely to have important roles in intramuscular homeostasis.[6,11] MSTN is a myokine responsible for regulating the growth and development of skeletal muscles.[12] It produces a balanced inhibition of muscle tissue to prevent uncontrolled proliferation and growth.[12] Quite conflicting data have been obtained regarding MSTN levels in response to exercise.[13]

Swimming is both a popular recreational activity and an effective sport to maintain and improve fitness.[14] Swimming exercises are also successfully applied in the treatment of some musculoskeletal and neurological diseases. It is different from other exercises in many ways, such as the environment, position, breathing pattern and muscle groups used.[15] Swimming being a natural behavior model for mice creates minimal stress and muscle damage thus can be preferred with these features.[16]

In the light of above information, the aim of this study was to determine blood concentrations of CNTF, CXCL-1, CT-1, OSM and MSTN myokines 3, 24 and 48 h after acute and chronic swimming exercise. In the international scientific community, there is a tendency to reveal myokine, cytokine-mediated physiological adaptations that develop following different types of exercise and to apply these to the individuals as drugs when necessary in the future.[5] Revealing blood concentrations of CNTF, CXCL-1, CT-1 OSM, and MSTN in response to acute and long-term swimming exercise may contribute to the mentioned effort. In addition, the data obtained may contribute to the development of new approaches for the prevention or treatment of a group of muscle diseases.

MATERIAL AND METHODS

ANIMAL CARE

Mice used in this study were obtained from Pamukkale University Experimental Animals Research

Unit. Prior to the study, approval was obtained from Pamukkale University Animal Research Ethics Committee (date: July 16, 2019; no: 05). The research was carried out in accordance with Ethics Committee regulations. The study was conducted in accordance with the principles of the Helsinki Declaration.

STUDY DESIGN

Twelve weeks old, 70 adult BALB/c male mice were used in the experiments. The animals were housed in specially prepared cages; under the control of a veterinarian, in a room with controlled temperature (23±2 °C) and relative humidity (50±5%) under a 12-h light cycle with free access to water and food. Standard mouse pellet food was used for feeding the mice. Tap water was provided as drinking water ad libitum. Since OSM, CT-1, CNTF levels are affected by inflammation, the mice were checked very strictly by a veterinarian. The experimental groups were planned as shown in Table 1. Mice were divided into control and swimming exercise groups. Control group was allowed to roam freely in their cages, but handling was applied daily. Exercising mice were further grouped as acute and chronic exercise, and each group was divided in terms of time (3 h, 24 h, 48 h) passed following the exercise until the end of the experiment. Thus, a total of 7 experimental groups were established.

Aerobic Swimming Exercise Protocol

A tank filled with water (68 cm x 44 cm x 38 cm) was used for swimming exercises. The temperature of the water was kept between 30 °C and 32 °C. Mice swam simultaneously in groups of 5 and 6. A 30 min session of acute exercise was applied, while chronic exercise groups swam 6 weeks, 5 days/week, 30 min/day. For adaptation, on the 1st day, the animals exercised for 10 min. The exercise period was prolonged 10 min every day until 30 min was reached. After each swimming exercise, the mice were dried with a towel and then placed in their cages. Mice of the control group were swum 10 min/week in the same tank and water in order to experience the same stress and water immersion.

Collection of Blood Samples

The experiments were termed by the exsanguination of the mice under ketamine-HCl/Xylazine-HCl (75 mg/kg-10 mg/kg) anesthesia. All blood was removed from the hearts of mice with a syringe. Blood samples collected in heparin-washed glass tubes were centrifuged and plasma were stored at -80 °C for later analysis.

ELISA Assay

Double antibody sandwich ELISA was used for the determination of plasma CNTF, CXCL-1, CT-1, OSM and MSTN levels. ELISA (Sunred Biological Technology, Shanghai, China) assays were performed and interpreted according to the manufacturers' instructions. Absorbance readings at 450 nm were performed using a micro plate reader (Sunrise, TECAN) to determine OD values.

STATISTICAL ANALYSES

It was observed that the effect size obtained in the reference study was quite strong (d=1.69). As a result of the power analysis, assuming that there will be 7 groups in the study and that a lower effect size can be achieved (f=0.6), it was calculated that 80%

TABLE 1: Representation of experimental groups.

1. Control group (n=9)		
2. Swimming exercise group	Acute 30 min	Exsanguination 3 h after the last exercise session (n=11)
		Exsanguination 24 h after the last exercise session (n=8)
		Exsanguination 48 h after the last exercise session (n=10)
	Chronic 6 weeks, 5 days/week, 30 min	Exsanguination 3 h after the last exercise session (n=10)
		Exsanguination 24 h after the last exercise session (n=9)
		Exsanguination 48 h after the last exercise session (n=13)

power could be obtained at 95% confidence level, when at least 49 mice were included in the study (at least 7 mice for each group). Considering that the amount of blood obtained may not be sufficient due to the fact that the mouse is a very small animal and the number of parameters, it was decided to carry out the experiments with 10 mice in each group and a total of 70 mice. Data were analyzed with SPSS 25.0 [IBM SPSS Statistics 25 software (Armonk, NY: IBM Corp.)] package program. Continuous variables were expressed as mean±standard deviation. The conformity of the data to the normal distribution was examined with the Shapiro-Wilk test. One-way analysis of variance was used to compare the independent group differences when parametric test assumptions were met; when the parametric test assumptions were not met, the Kruskal-Wallis analysis of variance test and then Mann-Whitney U test were used to compare independent group differences. $p<0.05$ was considered statistically significant.

RESULTS

Plasma CNTF, CXCL-1, CT-1, OSM and MSTN concentrations of control and exercise groups are demonstrated in Figure 1, 2, 3, 4 and 5, respectively. No statistically significant alterations in plasma levels of these myokines at 3, 24 and 48 h following acute and chronic swimming exercises were observed ($p=0.591$, $p=0.26$, $p=0.626$, $p=0.792$ and $p=0.681$, respectively).

FIGURE 1: CNTF concentrations of the experimental groups (pg/mL).
Mean ± SD. Control n=7, acute 3 h n=8, acute 24 h n=8, acute 48 h n=7, chronic 3 h n=8, chronic 24 h n=8, chronic 48 h n=10.

FIGURE 2: CXCL-1 concentrations of the experimental groups (pg/ml).
Mean ± SD. Control n=7, acute 3 h n=9, acute 24 h n=8, acute 48 h n=8, chronic 3 h n=9, chronic 24 h n=9, chronic 48 *h* n=11.

FIGURE 3: CT-1 concentrations of the experimental groups (ng/L).
Mean ± SD. Control n=7, acute 3 h n=9, acute 24 h n=8, acute 48 h n=8, chronic 3 h n=9, chronic 24 h n=9, chronic 48 h n=11.

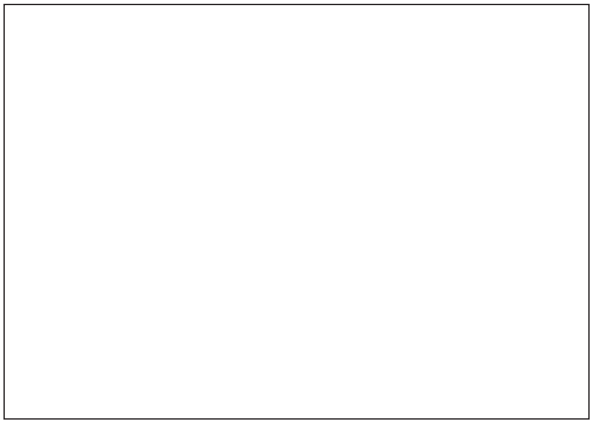

FIGURE 4: OSM concentrations of the experimental groups (ng/L).
Mean ± SD. Control n=8, acute 3 h n=9, acute 24 h n=8, acute 48 h n=8, chronic 3 h n=8, chronic 24 h n=9, chronic 48 h n=11.

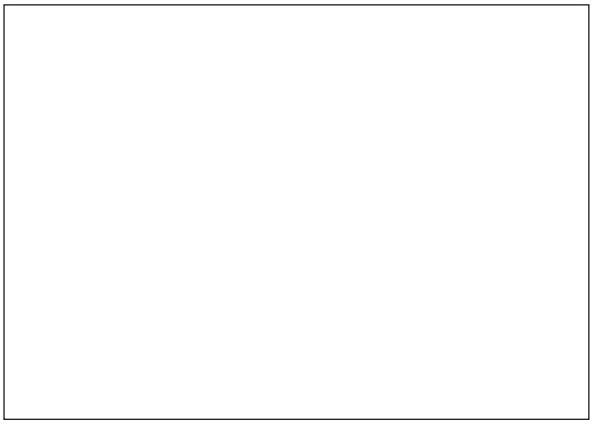

FIGURE 5: MSTN concentrations of the experimental groups (ng/L). Mean ± SD. Control n=7, acute 3 h n=9, acute 24 h n=8, acute 48 h n=7, chronic 3 h n=9, chronic 24 h n=8, chronic 48 h n=11.

DISCUSSION

Exercise not only has roles in the protection of functional well-being, but also reduces the risk of many diseases.[17] Preservation of skeletal muscle mass is critical in maintaining quality of life. Exercise has positive effects on muscle mass and functions, leads to an increase in muscle strength by increasing the sarcomere myofibril content and sarcomere number.[18] Myokines, which may be responsible for the beneficial effects of exercise are cytokines synthesized and released by myocytes during muscle contraction due to mechanical loading.[19] Muscle metabolism and tissues as adipose tissue, liver, and brain play role in the regulation of paracrine/endocrine responses to exercise.[19] It was predicted that revealing time-dependent changes in myokine levels in response to swimming exercise protocols may provide useful information for the development of new approaches to muscle damage and various muscle diseases. Thus, plasma levels of 5 different myokines (CNTF, CXCL-1, CT-1, OSM and MSTN) in response to acute and chronic (6 weeks) swimming exercise in a time-dependent manner (3, 24 and 48 h) were examined.

No statistically significant alterations in plasma CNTF, CXCL-1, CT-1, OSM and MSTN myokine levels at 3, 24 and 48 h following acute and chronic exercise sessions were observed. Plasma myokine levels reflect myokines released from the whole body. All myokine levels were determined using commercial kits. While choosing the kits, the most practical kits were tried to be preferred. Care was taken to select the best kits allowed by the study budget in terms of reproducibility and reliability of the results. However, while discussing the results, it is useful not to ignore the possibility of obtaining different results in case of using different commercial kits.

MSTN produced in skeletal muscle is a regulator of hypertrophy due to muscle overload.[20] It has been shown to suppress growth and differentiation in skeletal muscle. Considering age and gender, an inverse correlation was observed between serum MSTN levels and total body muscle mass.[21] In this study no significant changes was observed in plasma MSTN levels. Considering that MSTN levels were measured at 3, 24, and 48 h after exercise, it may be speculated that a correlation might be observed at other time intervals.

Studies examining MSTN responses to acute and long-term exercise have revealed different results depending on the type, intensity, duration, frequency of exercise and the time elapsed from exercise to tissue acquisition.[22] Two sets of acute swimming exercise for 3 h each was applied to a group of rats, while the other group swam 60 minutes, 5 days a week for 4 weeks.[8] Acute swimming exercise did not change MSTN expression in skeletal muscles, while chronic swimming exercise resulted in a significant decrease in MSTN mRNA expression.[8] In that study, it was suggested that MSTN expression is regulated in a way that supports skeletal muscle hypertrophy following chronic exercise.[8] Similar to our findings, no statistically significant difference was observed in skeletal muscle MSTN expressions following 4 weeks swimming (5 days a week, 1.5 h/day) in rats.[23] There are no studies examining the effects of acute and chronic swimming exercise on MSTN levels in mice. We did not determine mRNA expression of MSTN. If there is a stimulus threshold that reduces MSTN expression from skeletal muscle to suppress plasma MSTN levels, the 30-minute session or 6-week exercise period may have been insufficient to trigger it.

CNTF has been shown to play a role in the regulation of muscle regeneration after muscle injury in

rodents, and growth and development of peripheral nerves.[24] In addition, it has been reported that CNTF has anti-inflammatory properties and reduces food cravings.[25] In a recent study, serum CNTF levels were investigated in 94 female subjects diagnosed with multiple sclerosis by applying various exercise programs (12 weeks, 3 sessions per week) including resistance, endurance, pilates, balance and stretching exercises.[26] Consistent with our findings, none of the applied exercise programs affected serum CNTF levels.[26] Up to our knowledge, current study is the only one examining CNTF levels following swimming exercise.

It has been suggested that an increase in CT-1 in response to muscle contraction may mediate the beneficial effects of exercise through regulation of the metabolic (lipolysis and insulin-stimulated glucose uptake), cardiovascular and the immune systems.[4] In a human study, blood was collected from the individuals before exercise (single session resistance exercise with a bicycle ergometer), at the peak of exercise and following exercise. Similar to our results, no statistically significant alteration in CT-1 concentrations were detected in sedentary subjects.[4] In another study, treadmill (60 minutes a day, 3 days a week, 11 weeks) and swimming (60 minutes a day, 3 days a week, 11 weeks) exercises were applied to healthy rats and a chronic renal failure (CRF) model.[6] CT-1 level was significantly higher in the CRF group compared to the control group.[6] While CT-1 levels increased in healthy rats following both exercise protocols, a decrease in the CRF group was observed.[6] The incompability between our results and the above mentioned study, may be due to the type of the experimental animal used, the intensity/duration and frequency of swimming exercise and post-exercise blood collection times. Blood was drawn at 3, 24 and 48 h following the exercise in the current study, a possible alteration in plasma CT-1 levels between these periods might not have been detected.

OSM plays role in glucose, lipid and energy metabolism exerting its effects in muscle, liver, bone, brain, and inflammatory cells.[27] Protecting against tissue injuries, OSM is thought to work in exercise-related muscle damage, metabolism and remodeling.[28-30] There are no articles in the literature examining the OSM response to chronic exercise. When a single session of aerobic exercise (60 minutes) was performed, serum OSM concentrations have been shown to increase immediately after exercise in men.[31] Additionally, the serum OSM level, which was too low to be measured before exercise, was shown to increase immediately after a single session of acute swimming exercise (60 min). It has been shown to regress to its pre-exercise value in the 2nd h following the exercise.[7] We have measured plasma OSM levels 3, 24 and 48 h after acute and chronic exercise sessions and found no statistically significant alterations. Our data partly support the results of Hojman et al. who demonstrated that, exercise-induced rise in OSM levels fall to pre-exercise values in the 2nd h following exercise.[7]

Increment of CXCL-1 is known to cause macrophage invasion to the active muscle region.[32,33] Thus, exercise-induced CXCL-1 may function as a myokine responsible for leukocyte infiltration and muscle regeneration.[34,35] When acute swimming exercise (one session for 1 h) was applied to mice, serum CXCL-1 level increased significantly at the 2nd h following exercise and although a decrease was observed in the 5th h measurements after exercise, it was still significantly higher than the control group.[5] In the same study, it was reported that muscle CXCL-1 mRNA expression increased and working skeletal muscles stimulated the increase of liver CXCL-1 production.[5] The key point in muscle-liver communication in this study is that interleukin-6 released from the muscle in response to exercise binds to hepatocytes and increases CXCL-1 release.[5] These results belong to the only article in the literature examining CXCL-1 levels in response to acute exercise, and the change in CXCL-1 levels due to long-term exercise was not examined.

CONCLUSION

In conclusion, we have selected 5 myokines (CNTF, CXCL-1, CT-1, OSM and MSTN) due to their features summarized above and measured their plasma levels at 3, 24, and 48 h following both acute and chronic swimming exercise to determine their possible roles in exercise-induced adaptations. Although

no statistically significant alteration was detected in plasma levels of these myokines, this finding may not rule out that the aforementioned myokines may be playing role in adaptation to exercise. The main reason of getting the current results may be our post-exercise sample collection times. Due to the sample times we have chosen, we might have missed possible exercise-related myokine changes. The measurement methods of myokine levels might also have played a role. In addition, plasma myokine measurements may not be a complete indicator of the amount released from the exercising muscle, as they reflect the level throughout the body. Determining myokine levels and mRNA expressions in muscles used extensively during the exercise protocol may be a more appropriate approach. Myokines are a relatively new research topic. Other myokine levels and their post-exercise time-dependent alterations may also be examined to elucidate exercise-induced acute and chronic adaptations. Further studies are needed to clarify the issue.

Source of Finance

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Egem Burcu Ünal, Melek Bor Küçükatay;* ***Design:*** *Egem Burcu Ünal, Melek Bor Küçükatay;* ***Control/Supervision:*** *Melek Bor Küçükatay;* ***Data Collection and/or Processing:*** *Egem Burcu Ünal, Özgen Kılıç Erkek;* ***Analysis and/or Interpretation:*** *Egem Burcu Ünal, Melek Bor Küçükatay, Özgen Kılıç Erkek;* ***Literature Review:*** *Egem Burcu Ünal, Melek Bor Küçükatay;* ***Writing the Article:*** *Egem Burcu Ünal, Melek Bor Küçükatay;* ***Critical Review:*** *Melek Bor Küçükatay;* ***References and Fundings:*** *Egem Burcu Ünal, Özgen Kılıç Erkek, Melek Bor Küçükatay;* ***Materials:*** *Egem Burcu Ünal, Özgen Kılıç Erkek, Melek Bor Küçükatay;*

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