

Comparison of Meteorin-like Protein Infusion and Moderate-Intensity Exercise on Cardiac Mast Cell and Plasma Cell Dynamics and Musclin Levels in Female Rats

Dişi Sıçanlarda Kardiyak Mast Hücre ve Plazma Hücre Dinamikleri ve Musclin Seviyeleri Üzerinde Meteorin-Like Protein İnfüzyonu ile Orta Şiddetli Egzersizin Karşılaştırılması

ABSTRACT

Objective: Moderate-intensity exercise modulates the immunological response in cardiac tissue. Meteorin-like protein (METRNL) is a myokine secreted by muscle cells during exercise and is involved in immune response regulation. However, the effects of metrn1 on mast cells and plasma cells in cardiac tissue are not fully understood. This study was designed to assess the effects of exogenous metrn1 infusion on the cardiac mast cells and plasma cells. In addition, serum levels of musclin, an exercise-responsive factor, were evaluated during the effects of moderate-intensity exercise on cardiac immune cells.

Method: Twenty-seven female rats were randomly divided into three groups (n = 9 each): control (deionized water), exercise (moderate-intensity swimming exercise) and metrn1 (1 µg/day). For histological studies, hematoxylin-eosin, toluidine blue and methyl green-pyronin staining were performed on heart tissues. Musclin levels were measured in serum samples using the ELISA method.

Results: Metrn1 infusion increased cardiac mast cell and plasma cell numbers in female rats like moderate-intensity exercise. In addition, the increase in cardiac mast cell count was greater in the exercise group, whereas musclin concentration decreased in female rats subjected to moderate-intensity exercise.

Conclusion: Our data suggest that moderate-intensity exercise's effects on the cardiac immune system may be mediated by musclin downregulation and metrn1-dependent upregulation of cardiac mast cells and plasma cells. Thus, exercise-induced metrn1 may affect the cardiac immune response by modulating cardiac immune cells.

Keywords: Exercise, heart, mast cell, meteorin-like protein, musclin (osteocrin), plasma cell

ÖZET

Amaç: Orta şiddetli egzersiz kalp dokusunda immünolojik cevabı modüle eder. Meteorin-like protein (metrn1), egzersiz sırasında kas hücreleri tarafından salgılanan bir miyokindir ve bağışıklık yanıtının düzenlenmesinde rol oynar. Ancak, metrn1'in kardiyak dokuda mast hücre ve plazma hücresi üzerindeki etkileri tam olarak bilinmemektedir. Bu çalışma, ekzojen metrn1 infüzyonunun kardiyak mast hücre ve plazma hücresi üzerindeki etkilerini değerlendirmek için tasarlandı. Ayrıca, orta şiddetli egzersizin kardiyak bağışıklık hücreleri üzerindeki etkileri sırasında egzersize yanıt veren bir faktör olan musclinin serum seviyeleri değerlendirildi.

Yöntem: 27 adet dişi sıçan rastgele 3 gruba ayrıldı (n = 9, her grupta): kontrol (deiyonize su), egzersiz (orta şiddetli yüzme egzersizi) ve metrn1 (1 µg/gün). Histolojik çalışmalar için kalp dokularında hematoksilin-eozin, toluidine blue ve metil green-pironin boyaması yapıldı. Musclin seviyeleri serum örneklerinde ELISA yöntemi kullanılarak ölçüldü.

Bulgular: Dişi sıçanlarda metrn1 infüzyonu orta şiddetli egzersiz gibi kardiyak mast hücre ve plazma hücre sayılarını artırdı. Ayrıca, kardiyak mast hücre sayısındaki artış egzersiz grubunda daha fazlaydı, buna karşın orta şiddetli egzersize tabi tutulan dişi sıçanlarda musclin konsantrasyonu azaldı.

Sonuç: Özetle, verilerimiz orta şiddetli egzersizin kardiyak immün sistem üzerindeki etkilerine musclinin downregülasyonunun ve metrn1 bağımlı kardiyak mast hücre ve plazma hücrelerinin upregülasyonunun aracılık edebileceğini göstermektedir. Bu nedenle, egzersiz indüklü metrn1, kardiyak bağışıklık hücrelerini modüle ederek kardiyak immün yanıtını etkileyebilir.

Anahtar Kelimeler: Egzersiz, kalp, mast hücresi, meteorin-like protein, musclin (osteocrin), plazma hücresi

ORIGINAL ARTICLE KLİNİK ÇALIŞMA

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The immune system is crucial for preserving health and is an extremely complex organization of cells and molecules with specialized roles in defending against infection.¹ The innate and adaptive arms of immunity are in constant interaction and work in coordination with each other. The innate immune cells include granulocytes, macrophages, dendritic cells and mast cells, which respond rapidly, while adaptive immune cells, consisting of B and T cells, have a delayed response.² Mechanistically, it has been demonstrated that both innate and adaptive immunity have a part to play in cardiac physiology.³

Mast cells, regarded as one of the fundamental components of the immune system, are implicated in various disorders, including allergy, asthma, anaphylaxis, as well as gastrointestinal and cardiovascular diseases. In addition, mast cells also regulate a number of normal physiological processes such as vasodilation and angiogenesis.⁴ The bone marrow's pluripotent progenitor cells are the source of mast cells and mast cells are present in various organs and tissues such as skin, airways, gastrointestinal tract, testis, ovary and heart.⁵ Aside from mast cells, a distinct variety of immune cells, such as resident macrophages, patrolling monocytes, dendritic cells, T cells, B cells and plasma cells, are also present in the healthy, homeostatic myocardium.⁶

Terminal differentiation of B cells generates plasma cells that secrete large quantities of antibodies during humoral immune response. Thus, plasma cells are vital effector cells of humoral immunity. Found mostly in connective tissue and very rarely in the blood, long-lived plasma cells exist primarily in the bone marrow, but they are also found in various lymphoid organs throughout the body and, in disease states, in non-lymphoid organs.⁷

Systemic exposure to repeated exercise stimuli leads to long-term adaptations in various tissues, which triggers a number of well-known effects of exercise. These include greater vascularization and mitochondrial biogenesis, improved adipose and liver tissue processing of substrates, as well as improved cardiac and immune cell function.⁸ Based on the relationship between exercise, immunological response and health, it is known that exercise strengthens the immune system, by lowering oxidative stress linked to inflammatory marker levels and enhancing the body's immunological response.⁹ Exercise causes the immune system to undergo major physiological changes and these changes vary greatly depending on the nature and intensity of the exercise. Exercise-induced immunological alterations are caused by a variety of multifactorial pathways, including several neuroendocrine variables and immune cell function.¹ For example, the human immune system is greatly impacted by low-intensity physical exercise since it can greatly boost immune cell proliferation and inactivation capacity.¹⁰ It has been shown that an increased release of various myokines due to physical exercise, such as interleukin-6 (IL-6), can neutralize inflammation by attracting other immune cells and producing anti-inflammatory cytokines. At present, it has been proposed that myokines could mediate the health benefits of exercise and, in particular, that they might help prevent chronic diseases such as diabetes and cardiovascular disease, that are linked to low-grade inflammation.¹¹ However, despite the immunomodulatory effects of exercise, the fundamental mechanisms that support

ABBREVIATIONS

ANOVA	One-way analysis of variance
ELISA	Enzyme-linked immunosorbent assay
SEM	Standard error of the mean

cardiovascular health are not fully understood. Recognizing the key determinants by which exercise modulates immune function could potentially provide new therapeutic targets for the treatment of cardiovascular disease.

Meteorin-like protein (metrnl) has been identified as a myokine, adipokine and cardiokine expressed in a variety of tissues such as skeletal muscle, adipose tissue and heart. Metrnl is a key component of muscle metabolism, the maintenance of systemic energy balance and inflammatory immune regulations and is released by muscle cells during exercise. It has been reported that increased metrnl release resulting from moderate-intensity physical activity, reduced inflammation and pyroptosis. Accordingly, metrnl has been noted to have cardioprotective effects by affecting systemic inflammation.^{12,13} However, the effects of metrnl on mast cells and plasma cells in the heart tissue, regarded as an exercise mediator, are not fully known.

Musclin, another name for osteocrin, is a myokine that responds to exercise and a peptide belonging to the natriuretic peptide family. The expression of musclin varies depending on nutritional changes (like obesity), diabetes, cold exposure and exercise intervention. Accordingly, it has been shown that musclin levels are induced or downregulated by exercise. It has been demonstrated that musclin is involved in several metabolic processes, such as mediating insulin-dependent glucose metabolism, promoting mitochondrial biogenesis and regulating cardiovascular homeostasis, by attenuating inflammation.^{14,15} However, the relationship between the circulating musclin level and the mast cells and plasma cells, which are important components of the immune system during exercise, is not clear.

To investigate the possible role of exercise-responsive metrnl and musclin in the effects of moderate-intensity exercise on cardiac immune response, this study aimed to assess the effects of metrnl on the mast cell and plasma cell counts in the heart tissues and serum musclin levels, in comparison with moderate-intensity exercise in female rats.

Materials and Methods

This study was approved by the Animal Experimental Ethics Committee of Firat University (Approval Number: 2023/16-06, Date: 20.09.2023) and conducted in accordance with the Declaration of Helsinki. Artificial intelligence-assisted technologies were not used in the production of this study.

Animals

This study used exclusively female rats, since the immune system works more efficiently in females than in males.¹⁶ Estrous cycle monitoring was performed on 40 Sprague-Dawley rats (200–250 g, 2–3-month-old, obtained from Firat University's Experimental Animals Unit) for 10 days. At the end of the 10-day estrous cycle monitoring, a total of 27 rats determined to have regular estrous cycles were included in the study. The animals were housed

(three rats per cage) at a constant temperature (21 ± 1 °C) and humidity (50–55%) with a 12/12h light/dark cycle and food and water were provided ad libitum. The National Institutes of Health Guide for the Care and Use of Laboratory Animals was followed in all of the experiments.

Experimental Design

The animals were randomly split into three groups, each group containing nine rats: 1) Control group: Animals received intraperitoneal (i.p.) injections of deionized water for 19 consecutive days 2) Metrnl group: Animals were given the i.p. injections of metrnl in a concentration of 1 µg/day for about 19 days.¹⁷ 3) Exercise group: Animals were submitted to a moderate-intensity swimming exercise (30 minutes/day, 5 days/week) for about 19 days.¹⁸

Swimming Exercise Protocol

The exercise group was given a swimming exercise consisting of two stages: swimming adaptation and swimming exercise. All animals in the exercise group were first allowed to swim freely in a swimming tank for five minutes per day for one week, to ensure adaptation to the exercise before starting swimming training. After the swimming adaptation period was completed, the animals were given swimming exercise for 30 minutes per day, five days per week for an average of 19 days. During the experiment, all swimming exercises were performed at 13:00–15:00 in the swimming tanks, which were 25 cm in diameter, 60 cm in height and filled with water at 32 ± 1 °C. The control and metrnl groups remained sedentary during the experiments.

Metrnl Infusion

When the animals in the exercise group completed the swimming adaptation period and started swimming exercise, the i.p. injections of the control and metrnl groups were started. Rats in the metrnl group received i.p. a dose of 1 µg/day of metrnl (CSB-EP719323RA, CusaBio, Wuhan, China) at 13:00–14:00 for about 19 consecutive days. Metrnl was dissolved in deionized water and administered to each rat in a volume of 1 mL/kg. The control group received a daily i.p. injection of 1 mL/kg deionized water in a similar manner.

Sample Processing

From the sixteenth day of injections of deionized water/metrnl and exercise training until the nineteenth day, the animals were sacrificed by decapitation at the estrous stage without anesthesia, to collect trunk blood and heart tissue samples. The blood samples were centrifuged at 4,500 rpm for 5 min at 4 °C to extract the serum. The serum samples were then kept at – 80 °C until the day when enzyme-linked immunosorbent assay (ELISA) was done. For histological examinations, the heart tissues excised from all groups were fixed in a 10% formalin.

Measurement of Musclin

The serum musclin levels were measured using a commercial rat-specific ELISA kit from SunRed Biotechnology Company (Cat no: 201-11-4519, Shanghai, China), following the manufacturer's instructions. The ELISA plate was measured at 450 nm using the ELISA plate reader (Multiskan FC, Thermo Scientific, USA). As reported by the manufacturer, the assay sensitivity was 0.55 ng/mL and the assay range was 0.8–125 ng/mL for the musclin ELISA.

Histology Studies

After fixation, the heart tissues were routinely processed and embedded in paraffin. For routine histological examination, serial 5-µm-thick sections at 30-µm intervals were prepared on microscope slides from all of the experimental groups tissue blocks. The sections of heart tissues were stained with hematoxylin-eosin for general histomorphologic analysis. For histochemical determination of mast cells and plasma cells, 5 µm sections from these blocks were stained with toluidine blue and methyl green-pyronin, respectively. All the preparations were studied and photographed with an examination microscope (Nikon Eclipse 50i).

Sections of heart tissue were stained with toluidine blue (0.5%, pH:0.5; Sigma-Aldrich, CAS 92-31-9) for ten minutes in order to evaluate mast cell count.¹⁹ To ascertain the mast cell's numerical distribution in the tissue samples, ten areas of the heart tissue sections in all the experimental groups were chosen randomly and the mast cells were counted in each area, after which the results' arithmetic mean was calculated. This was done by counting mast cells using a 100-square ocular micrometer (eyepiece graticule) at a 40x magnification. All of the data was then converted into mast cell numbers inside a 1 mm² unit area.²⁰ This method was also used to determine the numerical distribution of plasma cells in 5-µm-thick heart sections stained with methyl green-pyronin (Histomed, BS-366, Lot: 102023.018) for 1.5 min.²¹

Statistical Analysis

Data was presented as mean \pm standard error of the mean (SEM). Following the Shapiro-Wilk test to confirm the data's normality, the one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test, was used to evaluate all of the data. The differences were considered statistically significant when P-value was < 0.05. The SPSS version 22.0 (IBM, Armonk, NY, USA) was used to perform the statistical analysis.

Results

The effects of exercise and metrnl on the histological changes in the heart tissues were analyzed by hematoxylin-eosin staining. Accordingly, as in the control group, normal cardiac histomorphology, including regular myocardial muscle fibers, intercalated discs and collateral connections was observed, in the exercise and metrnl groups. Nuclei were noted, centrally placed in myocytes and stained euchromatically (Figure 1).

As a result of examining the toluidine blue-stained heart tissue sections of each experimental group, mast cells in cardiac tissue were distinguished by their apparent metachromasia and determined to be round, oval or spindle-shaped in size. In most cells, centrally or eccentrically located nuclei were covered by granules. Mast cells were found to be located among the myocardial muscle fibers in cardiac tissue, chiefly in the epicardium and around the blood vessels (Figure 2A–C). As illustrated in Figure 2D, when compared to the control group, moderate-intensity exercise treatment significantly increased cardiac mast cell numbers in female rats (4.8 ± 0.4 and 7.89 ± 0.4 , respectively, $P < 0.001$). In addition, the cardiac mast cell numbers showed a significant increase in the metrnl group, compared to the control rats (6.64 ± 0.5 and 4.8 ± 0.4 , respectively, $P = 0.025$, Figure 2D).

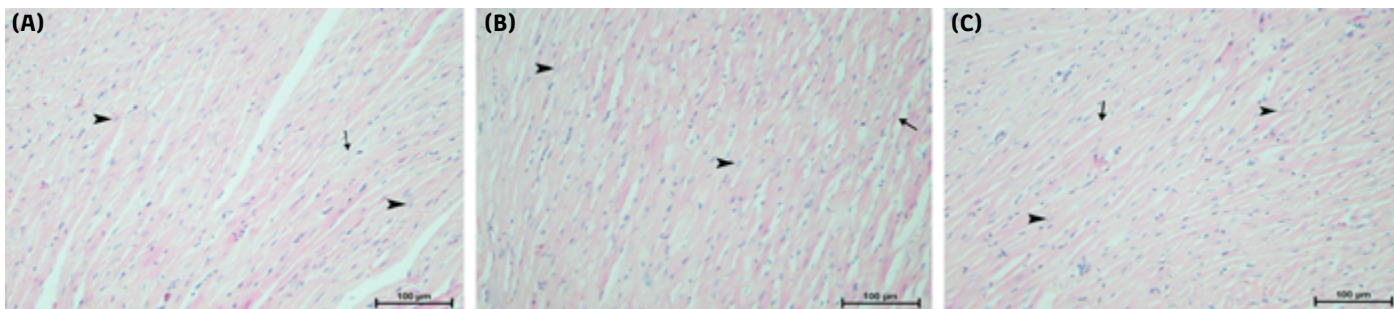


Figure 1. Histological slides of rat heart. (A) control group, (B) exercise group and (C) metrnl group (arrows indicating collateral connection and arrowheads indicating nucleus, hematoxylin and eosin stain, original magnification: 20x and scale bars: 100 μm).

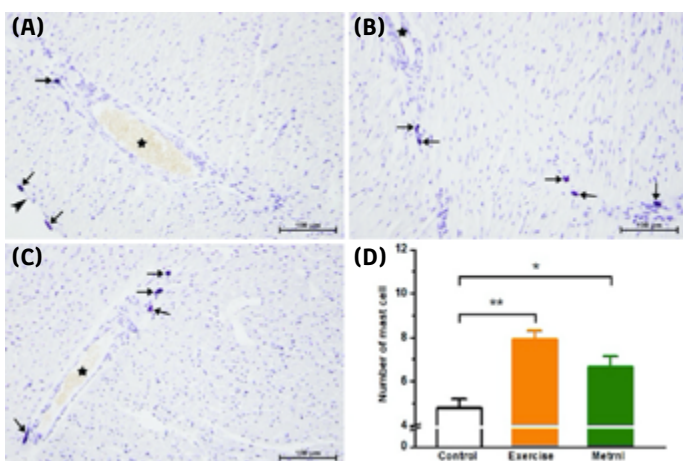


Figure 2. Effects of exercise or metrnl infusion on rat cardiac mast cells. Microscopic image of cardiac tissues in (A) control group, (B) exercise group and (C) metrnl group (arrows indicating mast cells, stars indicating blood vessels and arrowhead indicating pericardium, toluidine blue stain, original magnification: 20x and scale bars: 100 μm). (D) Mast cell counts (mean ± SEM), *P < 0.05, metrnl group vs. control group and **P < 0.001, exercise group vs. control group (one-way ANOVA followed by the Tukey's *post-hoc* test, n = 9 per group).

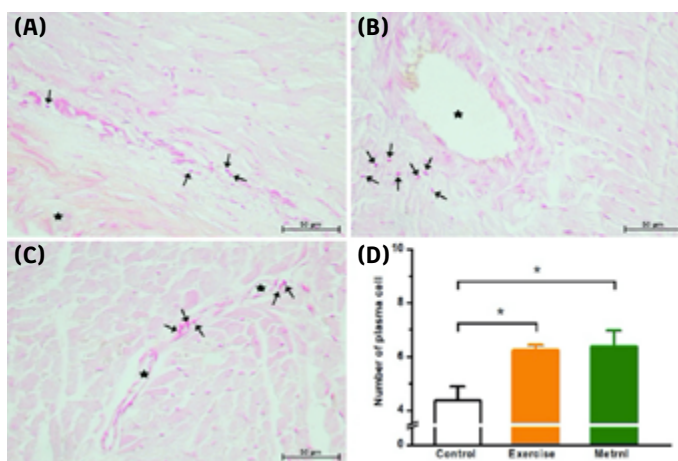


Figure 3. Effects of exercise or metrnl infusion on rat cardiac plasma cells. Microscopic image of cardiac tissues in (A) control group, (B) exercise group and (C) metrnl group (arrows indicating plasma cells and stars indicating blood vessels, methyl green-pyronin stain, original magnification: 40x and scale bars: 50 μm). (D) Plasma cell counts (mean ± SEM), *P < 0.05, metrnl or exercise groups vs. control group (one-way ANOVA followed by the Tukey's *post-hoc* test, n=9 per group).

In the heart tissues of all experimental groups, plasma cells stained with methyl green-pyronin were observed to be densely located in the epicardium and connective tissues around the blood vessels. It was determined that they were specifically found singly or in groups, around the blood vessels (Figure 3A-C). In the light microscopic examination, we found that plasma cell numbers in the heart tissues were significantly higher in the exercise group, in comparison to the control group (6.21 ± 0.2 and 4.37 ± 0.5 , respectively, $P = 0.049$, Figure 3D). As in the exercise group, metrnl infusion significantly increased plasma cell numbers in the heart tissues of female rats (6.37 ± 0.6 vs. 4.37 ± 0.5 compared to the control group, $P = 0.032$, Figure 3D). Furthermore, the data obtained also revealed that the number of plasma cells, as well as mast cells, in the cardiac tissues of female rats were similar between the metrnl and exercise groups (Figure 2D and Figure 3D).

The concentrations of serum musclin in female rats in the estrous phase are presented in Figure 4. Compared to the control group, serum musclin levels were significantly decreased, depending on

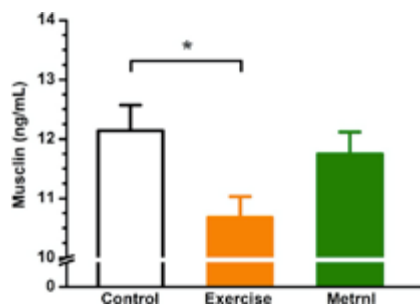


Figure 4. Effects of exercise or metrnl infusion on serum musclin levels in female rats. Data represent the mean ± SEM (n = 9 per group) *P < 0.05, exercise group vs. control group (one-way ANOVA followed by the Tukey's *post-hoc* test).

moderate-intensity exercise treatment in female rats (12.14 ± 0.43 ng/mL and 10.68 ± 0.35 ng/mL, respectively, $P = 0.038$). However, metrnl infusion did not affect the serum musclin levels in female rats (11.75 ± 0.37 ng/mL vs. 12.14 ± 0.43 ng/mL

compared to the control group, $P > 0.05$). Additionally, there was no significant difference in the serum musclin levels between the metrnl and exercise groups.

Discussion

In this study, we investigated the effects of metrnl, as an exercise-induced myokine, on mast cell and plasma cell numbers in the cardiac tissues of female rats, compared with moderate-intensity exercise. We also examined the changes in levels of the exercise-responsive myokine musclin during the effects of exercise and metrnl on the immune system cells. It was determined that metrnl increased mast cell and plasma cell numbers in the heart tissues like moderate-intensity exercise, but the rise in mast cell numbers was greater in female rats following moderate-intensity exercise. Additionally, serum musclin concentration in female rats was shown to decrease following moderate-intensity exercise and did not change with metrnl infusion. Accordingly, this study reported for the first time that exercise-induced metrnl may have a role in the immunoregulatory effects of moderate-intensity exercise in cardiac tissue, via immune cells.

The immune system is an intricate network of cells and molecules and it is affected by certain elements such as physiological factors, nutrition, environmental influences and exercise. Exercise causes physiological changes in the immune system by leading to changes in the number and function of immune cells.^{1,22} However, the positive and negative effects of exercise on the immune system vary depending on the exercise variables, including intensity, duration and type of exercise. It has been stated that moderate-intensity exercise is beneficial for the immune system via stimulating cellular immunity, while intense exercise can depress immunity by decreasing the number and activity of immune system cells, in healthy humans.^{23,24} For example, several studies have shown that moderate exercise caused greater increases in circulating immune cell counts, such as T and NK cells, than high-intensity exercise, and moderate-intensity exercise leads to stimulation of B cells.^{25,26} In another study, it was shown that exercise-induced cardioprotection was associated with increased cardiac myeloid-derived suppressor cells in mice, modelled with isoproterenol-induced heart failure.²⁷ Consistent with these findings, in the present study we observed that moderate-intensity exercise may have an effect on immunity, by causing an increase in cardiac mast cells and plasma cells of female rats.

Metrnl, an exercise-inducible protein, plays a role in inflammatory responses and has been associated with innate and acquired immune functions and inflammatory pathways. Metrnl has been shown to have a role in innate immunity, based on its much higher expression in the heart, skin, colon, trachea, tongue and other mucosal sites.²⁸ In addition, the fact that metrnl can be generated by thymic medullary epithelial cells, suggests that metrnl may impact T-cell development and thus, immune function.²⁹ The role of metrnl in the immune system is also consistent with previous reports showing that metrnl induces increased M2 macrophage polarization in adipose tissue, an increase in adipose tissue eosinophils and increased immune cell recruitment to injured skeletal muscle.³⁰ Consistently, our results showed that exercise-induced metrnl increased the numbers of mast cells and plasma cells in the heart tissues of female rats, just like moderate-intensity exercise treatment.

Moderate-intensity exercise decreased the risk for cardiovascular disease by altering immune cell function.³¹ Phungphong et al.³² in 2016 demonstrated that increased cardiac mast cell degranulation in the ovariectomized rat heart, was decreased with moderate-intensity exercise. Thus, they stated that one of exercise's cardioprotective mechanisms is through modulation of cardiac mast cell activation. Furthermore, recent studies have reported that metrnl, a myokine, adipokine and cardiokine, has potential cardioprotective effects in helping cardiac hypertrophy, dysfunction, postinfarction recovery and myocardial ischemia/reperfusion injury.^{13,33} However, current studies indicate that the underlying mechanism of metrnl in the cardioprotective effects remains unclear.³⁴

Mast cells, multifunctional effector cells of the immune system, are localized in various regions of the heart tissue, including the myocardium (i.e., coronary artery wall and aortic valves), endocardium and epicardium. Recent studies have revealed that mast cells in these different locations may contribute to both physiological and pathological processes. At present, it has been stated that myocardial mast cells may potentially exhibit cardioprotective effects by contributing to the prevention and recovery of ischemic damage to the myocardium.^{35,36} Studies have highlighted the potential benefits of bone marrow-derived B cells in improving cardiac function, after acute myocardial infarction. Similarly, Goodchild et al. in 2009 suggested that cardiac function was preserved by intramyocardial B cell injection into the early post-ischemic myocardium.^{37,38} Likewise, the present study revealed that plasma cells, which represent the final stage of differentiation for all antigen-activated B cells and mast cells, may have a central role in the cardiac immune responses of metrnl and exercise. Consequently, we speculated that metrnl may have a function in the moderate-intensity exercise-induced improvements in cardiac immune response, by altering cardiac mast cell and plasma cell dynamics. These new findings suggest that metrnl may have important clinical implications for cardiovascular health.

Skeletal muscle produces the peptide known as musclin, an exercise-stimulated myokine, in response to exercise, and musclin levels vary with exercise intervention. Studies have shown that musclin levels in the systemic circulation increase in mice due to treadmill exercise and similarly, the treadmill-based endurance exercise training increased the circulating concentration of musclin in humans. In contrast, it has been reported that swimming intervention reduces musclin expression in skeletal muscle in rats.^{14,39} Consistent with these findings, we observed that musclin levels decreased due to moderate-intensity swimming exercise in female rats. Additionally, for the first time in the literature, we report that exogenous metrnl infusion did not affect the musclin levels in female rats.

Regular, moderate-intensity exercise is linked with decreased circulating inflammatory cytokine levels, increased T-cell proliferation, greater natural killer cell cytotoxic activity and increased IL-2 production. All of these changes have been shown to indicate that regular, moderate-intensity exercise may improve or protect immunity.²⁴ Likewise, the current study determined that moderate-intensity exercise decreased the levels of the exercise-responsive myokine/cytokine musclin,

while increasing the number of cardiac mast cells, in female rats. Accordingly, this balance between the musclin levels and the number of mast cells due to moderate-intensity exercise, may be central in maintaining or improving immunity. In addition, the results of this study suggest that musclin may also be a candidate inflammatory cytokine in the cardiac immune response. Clinical implications of this study suggest that musclin level is a useful marker in the exercise-induced immune response.

Limitation of the Study

One potential limitation of our study is that it did not use immunohistochemical methods to identify mast cells and plasma cells in the cardiac tissue.

Conclusion

In conclusion, for the first time, our results show that metrnl increased mast cell and plasma cell numbers in cardiac tissue in female rats, like moderate-intensity exercise, but exercise intervention induced a greater increase in mast cell numbers and decreased musclin levels. Thus, it is suggested that the mechanisms underlying the modulation of the cardiac immune response of moderate-intensity exercise, are probably the effects of the exercise-responsive metrnl and musclin.

Ethics Committee Approval: Ethics committee approval was obtained from Animal Experimental Ethics Committee of Firat University (Approval Number: 2023/16-06, Date: 20.09.2023).

Informed Consent: Written informed consent was not required for this study.

Conflict of Interest: The authors have no conflicts of interest to declare.

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