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The effect of electrical stimulation of skeletal muscle on cardioprotection and on muscle-derived myokine levels in rats: A pilot study

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ABSTRACT

Electrical muscle stimulation (EMS) is a widely used method in sports and rehabilitation therapies to simulate physical exercise. EMS treatment via skeletal muscle activity improves the cardiovascular functions and the overall physical condition of the patients. However, the cardioprotective effect of EMS has not been proven so far, therefore, the aim of this study was to investigate the potential cardiac conditioning effect of EMS in an animal model. Low-frequency 35-min EMS was applied to the gastrocnemius muscle of male Wistar rats for three consecutive days. Their isolated hearts were then subjected to 30 min global ischemia and 120 min reperfusion. At the end of reperfusion cardiac specific creatine kinase (CK-MB) and lactate dehydrogenase (LDH) enzyme release and myocardial infarct size were determined. Additionally, skeletal muscle-driven myokine expression and release were also assessed. Phosphorylation of cardioprotective signaling pathway members AKT, ERK1/2, and STAT3 proteins were also measured. EMS significantly attenuated cardiac LDH and CK-MB enzyme activities in the coronary effluents at the end of the *ex vivo* reperfusion. EMS treatment considerably altered the myokine content of the stimulated gastrocnemius muscle without altering circulating myokine levels in the serum. Additionally, phosphorylation of cardiac AKT, ERK1/2, and STAT3 was not significantly different in the two groups. Despite the lack of significant infarct size reduction, the EMS treatment seems to influence the course of cellular damage due to ischemia/reperfusion and favorably modifies skeletal muscle myokine expressions. Our results suggest that EMS may have a protective effect on the myocardium, however, further optimization is required.

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KEYWORDS

electrical muscle stimulation, heart, cardioprotection, myokine, reperfusion injury

INTRODUCTION

Ischemic heart diseases including myocardial infarction are the leading cause of death worldwide [1]. It has been well established that there are remarkable endogenous adaptive responses of the heart to withstand the detrimental effects of ischemia/reperfusion (I/R) injury. Additionally, cardiac conditioning can be elicited through other organs termed as remote conditioning. Such interventions, which benefit multiple organs of the body at the same time, are of great clinical importance [2, 3].

The lack of physical exercise represents a global public health problem particularly because the human body rapidly adapts to insufficient physical activity [4]. The cardio-preventive and therapeutic potential of long-term exercise is manifested in lowering blood pressure, favorably modifying plasma lipoprotein profile, and enhancing cardiac contractile function. Apart from the advantageous outcome of exercise, the enormous adaptation capacity of the skeletal muscle might be transferred to the heart thereby inducing cardiac conditioning and subsequently protecting against the detrimental effects of I/R injury and having favorable outcomes in cardiac rehabilitation. However, performing regular exercise is often limited due to various health reasons. For those, electrical muscle stimulation (EMS), the rhythmical muscle activation triggered with electrical impulses, might provide an alternative way to partially gain the benefits of exercise [5, 6]. EMS is a widely used method in sport and rehabilitation therapy and is an attractive clinical application for subjects unable to perform regular exercise [7, 8]. Nevertheless, little is known whether the application of EMS can trigger cardiac preconditioning and protection against I/R injury.

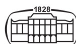
Both humoral and neurological factors are implicated in the advantageous effects of skeletal muscle activity on the general health of the individual. Recently, skeletal muscle-derived myokines are emerged as important effectors of exercise-induced cardioprotection [9]. Myokines are a wide variety of molecules predominantly released by contracting skeletal muscles, supposed to regulate muscle mass and energy homeostasis [10]. Beyond the effect on the skeletal muscles, myokines are also considered as molecular mediators which link muscle exercise to the whole body physiology through endocrine signaling pathways [11, 12].

Taken together the promising therapeutic potential of EMS, the aim of the present study was to test (i) whether short-term EMS confers cardioprotection as a remote preconditioning intervention and (ii) whether EMS modulates myokine levels in the stimulated skeletal muscle.

MATERIALS AND METHODS

Animals

Altogether 20 male Wistar rats (300–350 g) were used in this study. The animals were kept in pairs in individually ventilated cages in a temperature-controlled room with 12 h:12 h light/dark cycles. Laboratory chow and water were available ad libitum throughout the study. The experiment conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, re-vised 1996) and was approved by the Animal Research Ethics Committee of the University of Szeged (approval number: XV./2153/2022).



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Experimental setup

EMS treatment was performed in sedated animals with stimulating electrodes placed on the *gastrocnemius* muscles of the animals. EMS sessions were applied for three days; each session in each days includes 10-Hz frequency continuous stimulation for 35 min. Twenty-four hours after the last EMS treatment, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; Produlab Pharma b.v., Raamsdonksveer, The Netherlands), and blood and *gastrocnemius* muscle samples were collected for myokine level measurement. Hearts from EMS-treated and untreated animals were isolated and perfused according to Langendorff as described previously [13, 14]. A 15-min equilibration period was applied followed by 30 min global ischemia and 120 min reperfusion (I/R). Global ischemia was achieved via the complete closure of perfusion fluid toward the heart, therefore, the same degree of stress (i.e. ischemia) was applied to the entire myocardium. During reperfusion, coronary effluents were collected and used to measure cardiac lactate-dehydrogenase (LDH) and creatine kinase MB isoform (CK-MB) release. At the end of the reperfusion, infarct size was determined by TTC staining [15].

Electrical muscle stimulation

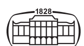
Rats were sedated with a 40 mg kg^{-1} sodium pentobarbital solution (Produlab Pharma b.v., Raamsdonksveer, The Netherlands), placed on a heating pad in a supine position, and hind limbs were fixed. EMS treatment was performed with a portable electrostimulator device (Sanitas SEM 44 digital EMS/TENS, Hans Dinslage GmbH, Germany) for three consecutive days. The stimulating pads of the EMS device were replaced with acupuncture needles to easily access the skeletal muscle with the electrodes. Bilateral EMS was applied targeting the gastrocnemius muscles with the stimulating electrodes once a day for three consecutive days with low frequency as reported in human studies [7, 16], respectively. The EMS treatment consisted of 35 min of continuous stimulation with bipolar rectangular pulses at 10 Hz frequency and 250 µs pulse width with minimal intensity to produce a visible muscle contraction. The control group underwent the same procedure, without switching on the EMS device.

Infarct size determination

Myocardial infarct size was measured after *ex vivo* global I/R in hearts isolated from both EMS-treated and untreated animals. After the end of reperfusion, atria were removed, and the total ventricles were used to determine the infarcted area. Briefly, frozen ventricles were cut into 7–8 equal slices and placed into triphenyl tetrazolium chloride (TTC) solution (Sigma, Saint Louis, MO, USA) for 10 min at 37 °C followed by a 10 min formaldehyde fixation and phosphate buffer washing steps [15]. As a result, survived area was stained red while the necrotic area remained pale. Digitalized images from the stained heart slices were evaluated with planimetry method and the amount of myocardial necrosis was expressed as infarct size/area at risk %.

LDH and CK-MB release measurement

Coronary effluents were collected 2, 5, 30, and 120 min after the beginning of reperfusion to measure the release of LDH and CK-MB enzymes. Enzyme activity was measured via kinetic enzyme activity assay using colorimetric LDH and CK-MB detecting kits (Diagnosticum, Hungary) and a microplate reader (Clariostar Plus, BMG Labtech). Enzyme activity was



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normalized to the volume of coronary effluent and the total weight of the respective heart. Enzyme release was expressed as U min^{-1} g^{-1}wet weight.

Western blot

In a separate set of experiments, left ventricles of isolated hearts from EMS-treated and untreated animals were homogenized with an ultrasonicator in Radio Immunoprecipitation Assay (RIPA) buffer (Cell Signaling, Danvers, MA, USA) supplemented with protease inhibitor cocktail and phosphatase inhibitors phenylmethane sulfonyl fluoride (PMSF) and sodium fluoride (NaF, Sigma, Saint Louis, MO, USA). Homogenates were centrifuged, and protein concentrations of the supernatants were determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Twenty μg of reduced and denatured protein was loaded in 10% polyacrylamide gel, and SDS gel electrophoresis was performed. Separated proteins were transferred to 0.22 μm pore size nitrocellulose membranes. After checking the transfer efficiency with Ponceau-staining, membranes were blocked for 1 h in 5% (w/v) bovine serum albumin (BSA) at room temperature. Blocked membranes were incubated with the following primary antibodies in the concentrations of 1:1000 phospho-AKT (Ser473, #4060), AKT (#9272), phospho-ERK1/2 (Thr202/Tyr204, #9101), ERK1/2 (#9102), phospho-STAT3 (Tyr705, #9145), STAT3 (#4904) and in 1:5000 concentration against GAPDH (#2118) at 4 °C overnight (Cell Signaling, Danvers, MA, USA). After incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody membranes were developed with an enhanced chemiluminescence kit. After the development of phosphorylated signals of AKT, ERK1/2, and STAT3 membranes were stripped and reassessed for the amount of total proteins. Signals were analyzed and evaluated by Quantity One Software.

ELISA

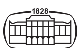
Double-antibody sandwich ELISA kits specific for rat Irisin, Decorin, Myonectin, Myoglobin, IL6, IL15, and FSTL1 proteins, respectively, were used to measure protein content in gastrocnemius and serum samples (Fine Test, Wuhan, China) according to the manufacturer's instruction. Myokine content was determined with a colorimetric detection method using a microplate reader (Clariostar Plus, BMG Labtech, Germany).

RT-qPCR

RNA was isolated from the *gastrocnemius* muscles with the phenol:chloroform:isoamyl alcohol extraction method and total RNA concentrations were determined. Reverse transcription of 500 ng RNA was performed with iScript cDNA Synthesis Kit (BioRad) and the resulting cDNA was used as a template for qPCR measurement of myokine expression levels using SYBR Green PCR Super Mix (BioRad) and BioRad CFX96 Touch Real-Time PCR machine (BioRad). Relative expression levels were determined with the $2^{-\Delta\Delta Ct}$ method and GAPDH was used as a housekeeping gene. Primer sequences are listed in Table 1, while primers used for the measurement of *Dcn, Fndc5,* and *Il15* are designed by BioRad (#10025636), and their relative expression was normalized for *Gapdh* provided by the same manufacturer.

Statistics

All values are expressed as mean ± SEM. The Shapiro-Wilk normality test was used to test the normal distribution of the data. Student's *t*-test was used to evaluate the effect of EMS on infarct



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size. Repeated measures ANOVA was applied for changes in mean scores over different time points of reperfusion for the evaluation of LDH and CK-MB releases. Relative expression levels of myokines at the transcript level were determined with the $2^{-\Delta\Delta Ct}$ method. For all statistical evaluation P value <0.05 was considered as an indicator of significant difference among the groups.

RESULTS

Testing the cardioprotective effect of skeletal muscle EMS treatment on *ex vivo* perfused hearts

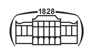
To assess the cardioprotective effect of EMS, *ex vivo* heart perfusion was performed on isolated hearts from EMS-treated and untreated control rats. LDH and CK-MB enzyme release, cardiac markers of the myocardial infarction, were measured from the coronary effluents collected at different time points of the reperfusion (Fig. 1A and B). Based on our results both cardiac LDH and CK-MB release were significantly lower upon EMS at the end of reperfusion. Furthermore, myocardial infarct size was also determined at the end of reperfusion, and although the mean value of infarct size tended to be lower in the EMS group compared to the nonstimulated control group, the applied EMS treatment failed to attenuate infarct size significantly (Fig. 1C).

Altered myokine expression in the gastrocnemius muscle in response to EMS

To determine the possible mediators of EMS-associated cardioprotection, myokine expression was measured in the stimulated gastrocnemius muscles. Among the investigated myokines the applied EMS treatment upregulated *Fstl1*, *Il6*, and *Igf1* mRNA levels in the gastrocnemius muscle (Table 2). Additionally, *Il15* mRNA content was downregulated in response to EMS.

Table 1. **Primer sequences used for the determination of myokine expression levels in the gastrocnemius muscle**. Fwd: forward, Rev: reverse primer

Fstl1 Fwd	GGCCTGTGTGTGGCAGTAAT	Follistatin-like 1
Fstl1 Rev	CAGCTCATCACGGTTAGCCT	
Fgf21 Fwd	CTCCAGTTTGGGGGTCAAGT	Fibroblast growth factor 21
Fgf21 Rev	GGAGACTTTCTGGACTGCGG	
Lif Fwd	AGTTGGTCGAGCTGTATCGG	Leukemia inhibitory factor
Lif Rev	GCCCACATGGTACTTGTTGC	
Il6 Fwd	GAGTTCCGTTTCTACCTGGAGT	Interleukin-6
Il6 Rev	TTGGTCCTTAGCCACTCCTTC	
Erfe Fwd	TCAAGCAGAGTGACAAGGGC	Erythroferrone/myonectin
Erfe Rev	CGTACCGCACCTTTCAACAA	
Bdnf Fwd	TCCCGGTATCAAAAGGCCAA	Brain-derived neurotrophic factor
Bdnf Rev	ATGAACCGCCAGCCAATTCT	
Igf1 Fwd	CTGGTGGACGCTCTTCAGTT	Insulin-like growth factor 1
Igf1 Rev	CGGATGGAACGAGCTGACTT	
Gapdh Fwd	GGTCATCAACGGGAAACCCA	Glyceraldehyde-3-phosphate dehydrogenase
Gapdh Rev	GAAGGGGCGGAGATGATGAC	



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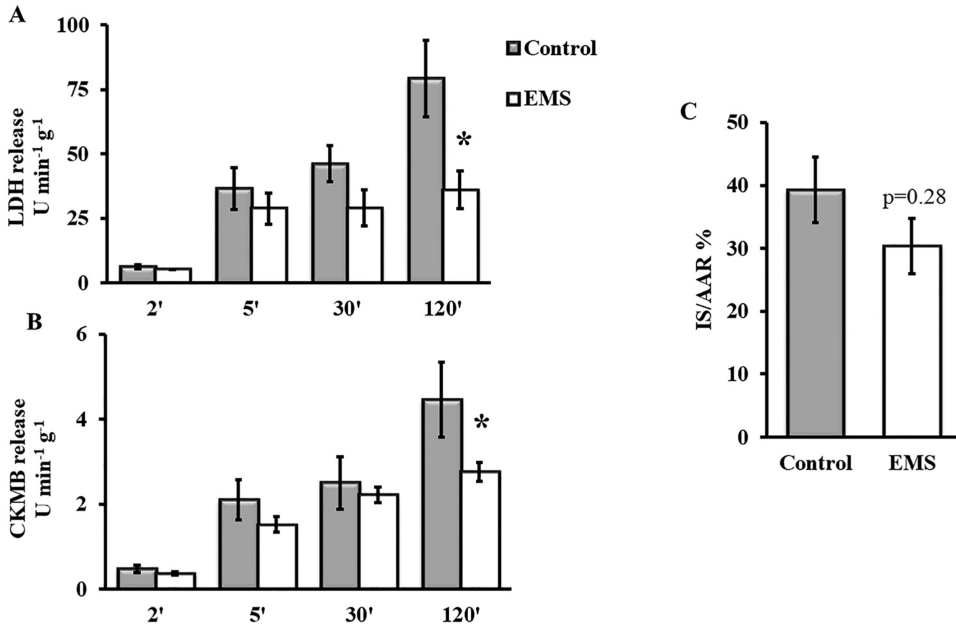
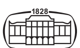


Fig. 1. Testing the potential preconditioning effect of EMS treatment against I/R in *ex vivo* perfused hearts. LDH (A) and CK-MB (B) enzyme release measurement. Coronary effluents were collected at different time points of the reperfusion and used for colorimetric CK-MB and LDH enzyme activity measurement. Infarct size values at the end of *ex vivo* heart perfusion (C). Hearts isolated from EMS-treated and untreated rats were subjected to 30 min global ischemia and 120 min reperfusion (I/R). Data are expressed as mean ± SEM; $n = 6$ in each group. $^{*}P < 0.05$

Table 2. **Myokine mRNA expression in the gastrocnemius muscle after EMS treatment**. *Fstl1*: Follistatin-like 1, *Fgf21*: Fibroblast growth factor 21, *Il6*: Interleukin-6, *Bdnf*: Brain-derived neurotrophic factor, *Erfe*: Erythroferrone/myonectin, *Igf1*: Insulin-like growth factor 1, *Lif*: Leukemia inhibitory factor, *Dcn*: Decorin, *Fndc5*: Fibronectin type III domain-containing protein 5/Irisin precursor, *Il15*: Interleukin-15, respectively. Data are expressed as mean ± SEM; $n = 10$, $^{*}P < 0.05$

Myokine mRNA	relative mRNA expression levels	
	Control	EMS
Fstl1	1.02 ± 0.08	2.80 ± 0.59 *
Fgf21	1.55 ± 0.47	1.44 ± 0.38
Il6	1.11 ± 0.17	3.90 ± 1.10 *
Bdnf	1.10 ± 0.15	0.93 ± 0.19
Erfe	1.07 ± 0.16	0.98 ± 0.11
Igf1	0.93 ± 0.07	1.42 ± 0.19 *
Lif	1.91 ± 0.70	1.50 ± 0.61
Dcn	1.08 ± 0.11	1.31 ± 0.21
Fndc5	1.06 ± 0.12	0.92 ± 0.15
Il15	1.04 ± 0.09	0.56 ± 0.07 *



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Next, we assessed the protein content of some selected myokines in the stimulated gastrocnemius muscle. Based on our results Irisin, Decorin, Myonectin, FSTL1, and Myoglobin proteins were upregulated as a consequence of EMS (Table 3). Nevertheless, IL-6 and IL-15 protein levels remained unaffected upon EMS treatment, despite the increased level of their mRNA transcripts.

Serum myokine levels remained unaffected upon EMS treatment

To elucidate whether skeletal muscle-derived myokines may contribute to the cardioprotective effects of EMS, serum myokine levels were measured by ELISA. However, at the time of serum sampling none of the measured myokines showed significant differences in the blood compared to the untreated control animals (Table 4).

Investigation of the possible involvement of cardiac conditioning-associated pathways upon EMS of the gastrocnemius muscle

To further clarify the possible cardioprotective effect of the skeletal muscle EMS, the key protein elements of the Reperfusion Injury Salvage Kinase (RISK) and the Survivor Activating Factor

Table 3. **Myokine protein levels in the stimulated gastrocnemius muscle determined with ELISA.** Protein concentration values are expressed as ng mg^{-1} tissue protein. FSTL1: Follistatin-like 1, IL-6: Interleukin 6, IL-15: Interleukin-15. All data are mean ± SEM; $n = 8$, $^{*}P < 0.05$

Myokine	Tissue protein content ng mg^{-1}	
	Control	EMS
Irisin	22.14 ± 3.52	39.59 ± 5.26*
Decorin	0.24 ± 0.05	0.55 ± 0.09*
Myonectin	2.13 ± 0.32	4.84 ± 0.94*
FSTL1	25.46 ± 2.52	33.00 ± 1.87*
IL-6	9.28 ± 0.89	10.61 ± 1.12
IL-15	0.97 ± 0.08	0.94 ± 0.08
Myoglobin	0.57 ± 0.10	1.31 ± 0.34*

Table 4. **Serum myokine levels after one day of the last EMS treatment**. Protein concentration values expressed as ng mg^{-1} serum protein FSTL1: Follistatin-like 1, Data are mean ± SEM; $n = 8$, $^{*}P < 0.05$

Myokine	Serum protein content	
	Control	EMS
Irisin	0.61 ± 0.06	0.62 ± 0.10
Decorin	0.011 ± 0.006	0.012 ± 0.013
Myonectin	2.22 ± 0.21	2.55 ± 0.31
FSTL1	0.25 ± 0.02	0.26 ± 0.03
Myoglobin	6.64 ± 0.80	6.95 ± 0.84

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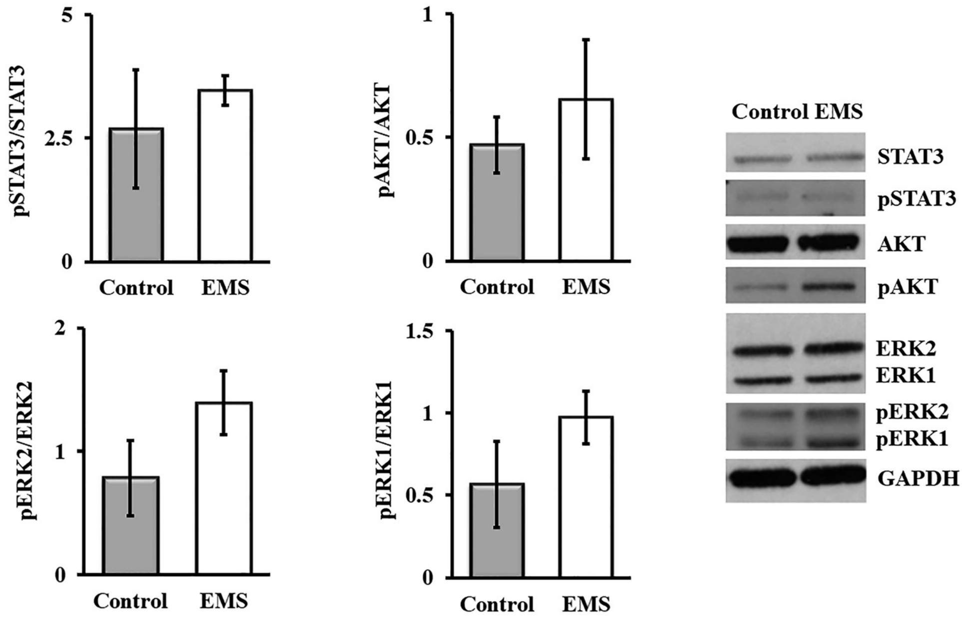


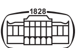
Fig. 2. Effect of skeletal muscle EMS training on the phosphorylation of STAT3, AKT and ERK1/2 proteins assessed by Western blots. Data are expressed as mean ± SEM; $n = 4$, $^*P < 0.05$

Enhancement (SAFE) pathways were investigated in ventricular samples at the end of the reperfusion. In regard to the RISK pathway, phosphorylation of AKT kinase was not affected significantly upon EMS, while phosphorylation of ERK1 and ERK2 showed a trend toward an increase ($P = 0.18$ and $P = 0.21$, respectively) in the hearts of EMS-treated animals compared to the untreated controls (Fig. 2). Additionally, phosphorylation of STAT3, the key transcription factor of the SAFE pathway, was not affected in the left ventricles either.

DISCUSSION

In the present study, we investigated whether EMS of the skeletal muscle confers cardioprotection in a remote preconditioning-like manner as well as if skeletal muscle myokine production and secretion is modified upon EMS treatment. Despite the lack of significant infarct size reduction, the EMS treatment seems to influence the course of cellular damage due to I/R shown by the decreased cardiac markers LDH and CK-MB release. Additionally, EMS altered the expression levels of several myokines in the skeletal muscle, which might be potential mediators of the beneficial effects of EMS in the cardiovascular system.

EMS has been long utilized to either supplement or substitute muscle strengthening in several rehabilitation settings [17, 18]. Although inferior to conventional training, nevertheless, improvements in exercise capacity and quality of life are established outcomes of EMS treatment in patients unable to perform regular exercise [7, 16, 19, 20]. The aim of the present study was to



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test whether a short duration of EMS treatment of rats protects their heart against *ex vivo* I/R injury. In our perfusion model LDH and CK-MB release during the reperfusion were significantly decreased in the *ex vivo* perfused hearts of animals that received EMS. However, EMS prior to global I/R failed to significantly decrease the infarcted area. Our results suggest that despite the lack of significant infarct size reduction EMS treatment might initiate different cardioprotective mechanisms which were partially retained during the *ex vivo* heart perfusion. The effect of electrical stimulation as a cardiac remote preconditioning maneuver was tested in previous studies with different experimental setups. In an early study, Birnbaum and colleagues [21] reported that in combination with remote ischemic preconditioning electrical stimulation of the gastrocnemius muscle conferred cardioprotection in a rabbit model of *in vivo* I/R, however, the same approach failed to enhance the efficiency of preconditioning of human patients underwent coronary angioplasty [22]. Nevertheless, previous research mainly focused on the direct electrical stimulation of peripheral nerves rather than the muscle contraction-mediated preconditioning effects. Targeted electrical stimulation of the medial nerve [23], the femoral nerve [24–26], or electroacupuncture pretreatment [27, 28] straight before I/R mitigated myocardial infarct size and improved the post-ischemic cardiac performance in rodents. These observations implicated the immediate release of small molecular weight blood-borne humoral factors upon electrical stimuli and subsequent cardiac opioid receptor agonism.

Several hundred cytokines, other small proteins, proteoglycans, and oligopeptides are produced and released by muscle in response to muscle contractions [29]. These molecules, termed myokines or exerkines, exert their effects on other organs through inflammation modulation or metabolic control. The effect of EMS on skeletal muscle myokine levels was also addressed in our current study. Based on our results, the applied EMS protocol increased *Il6* mRNA, but not IL-6 protein levels in the gastrocnemius muscle. Similarly to our findings, electrical stimulation of rat myotube culture evoked an increase in *Il6* mRNA levels [30]. Additionally, a short duration of neuromuscular electrical stimulation led to markedly increased circulating IL-6 levels in healthy individuals [31, 32]. In the skeletal muscle, the exercise-induced release of IL-6 seems to exert anti-inflammatory actions [32] and could act as one of the major mediators of exercise-induced cardioprotection against myocardial I/R injury [33]. In contrast with observations of exercise-mediated IL-15 response, in our experimental settings, EMS decreased *Il15* mRNA expression in the stimulated gastrocnemius muscles, without any effect on muscle IL-15 protein content. During exercise, IL-15 is one of the expressed cytokines in the muscle, with essential roles in skeletal muscle anabolic processes. Moreover, these data suggest a possible link in the exercise-mediated muscle-fat crosstalk [34, 35] and indicate potential anti-inflammatory effects [36]. However, based on our results, the applied EMS seems to have no effect on IL-15 production.

Another myokine, FSTL1 was the only myokine upregulated both in mRNA and protein levels in the muscle, without any change in the bloodstream upon EMS. FSTL1 is a glycoprotein, secreted by skeletal muscle in a contraction-regulated manner [37, 38]. However, the effect of EMS on skeletal muscle FSTL1 production and secretion has not been clarified yet. Görgens and colleagues found that acute exercise-induced FSTL1 expression is possibly regulated by inflammatory cytokines rather than electrical pulse stimulation in myoblast culture [39]. However, opposite scenario was observed in mesenchymal stem cell culture after short-term electrostimulation and it might be associated with cardiomyogenic differentiation [40]. Furthermore, FSTL1 has been described as a cardioprotective myokine [41–43]. The related beneficial effects

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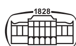
on the myocardium are shown to involve improved endothelial cell function, mitigation of myocardial ischemic injury, and revascularization in ischemic tissue [44, 45].

In contrast to the observed myokine mRNA expression levels, EMS induced an increment in the protein levels of Decorin, Irisin, Myonectin and Myoglobin, respectively, after three days of the stimulation period. However, none of the selected myokines showed altered levels in the serum of EMS-treated animals. All of the selected myokines were previously implicated with exercise-mediated secretion and protection against various ischemic injury-related cell death [9, 46, 47]. To the best of our knowledge, Decorin, Irisin, and Myonectin levels were not yet investigated upon EMS treatment and the disclosure of their paracrine and endocrine functions might provide great value to the better understanding of the systemic effects of involuntary contractions of the skeletal muscles elicited by electrical stimulations. However, apart from the myokines of the scope of our research, previous studies highlighted the local and systemic effects of many other electrical stimulation-driven myokine production and secretion [48]. Altered circulating myokine levels upon neuromuscular electrical stimulation treatment, notably, brain-derived neurotrophic factor (BDNF), meteorin-like (METRNL), and IGF-1, respectively, possibly exert beneficial metabolic effects on glucose metabolism of the diabetic patient, skeletal muscle anabolic processes as well mitigation of sarcopenia in elderly people [49–52]. Previous studies suggested the importance of intact peripheral nerves for triggering electrical stimulation-driven cardioprotection. However, myokines might overcome the damaged peripheral sensory nerves which seem to be mandatory for remote conditioning, which further strengthens their prominent role in the potential protection of the myocardium during the application of EMS [53].

In the present study, the possible activation of the cytosolic protein components of the cardiac preconditioning-associated mechanisms were also investigated. Based on our results, EMS treatment failed to significantly modify the phosphorylation of ERK1, ERK2 and AKT kinases, respectively. Furthermore, the key protein component of the SAFE pathway was also unaffected upon EMS of the gastrocnemius muscles as shown in the phosphorylated state of STAT3 in the left ventricle. Upon phosphorylation, the activation of the STAT3, AKT and ERK1/2 kinases share a crucial role triggering cardiac conditioning accompanied by protection against ischemic injury. Similarly to the classical ischemic preconditioning maneuver, signal transduction of remote ischemic preconditioning through skeletal muscles or regular physical activity led the activation of the RISK and SAFE as well the endothelial nitric oxide synthase/protein kinase G (eNOS/PKG) pathways, respectively, as reviewed elsewhere [54, 55]. Interestingly, previous findings of Tsai and colleagues [56] revealed that electrical stimulation of the median nerve induced phosphorylation of myocardial AKT, GSK-3, and PKCε proteins, respectively, suggesting the potential remote preconditioning ability of EMS on the heart via a yet-to-be confirmed mechanisms.

LIMITATIONS

We are aware that the present study is not without limitations. In order to test the potential cardioprotective effect of EMS, we applied an *ex vivo* heart perfusion system, where global ischemia was induced. This approach is different from the stress during *in vivo* occurring ischemia and reperfusion. As known, the second window of preconditioning may be less robust than that of the first window, which requires further consideration of the exact time of testing the beneficial effects of EMS against I/R on the heart. However, the potential role of myokines on EMS-induced remote protection was also assessed, which molecules possibly contribute in a



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later phase of the protection. Moreover, measuring secondary endpoints, like the incidence of myocardial arrhythmias might also be helpful to determine the preconditioning effect of EMS. Myokine levels were only measured one day after the third EMS session, which could also hide changes upon EMS due to the fast turnover of circulating proteins. Therefore, different sampling times for serum myokine measurements, as well as assessment of previously described myokine-evoked molecular changes in the heart, might elucidate the conditional role of several myokines in EMS-induced cardioprotection. Additionally, due to the anatomical characteristics of rats, EMS treatment was only applied to the gastrocnemius muscle, which is easily accessible for surface electrodes. Nevertheless, a more robust change in circulating myokine levels might happen with the involvement of greater muscle mass.

CONCLUSIONS

Despite the lack of significant infarct size reduction, the EMS treatment seems to influence the course of cellular damage due to ischemia/reperfusion. Our results suggest that EMS may have a protective effect on the myocardium, however, further optimization of the protocol is required. Similarly to voluntary exercise, myokines might be also involved in the cardioprotective effects of EMS, however, verification of their mechanistic role remains for the scope of future studies.

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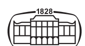

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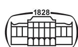

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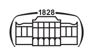

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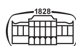

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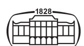

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