Sympathetic Nerve Innervation and Metabolism in Ischemic Myocardium in Response To Remote Ischemic Conditioning

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Research Article

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Abstract

Background

Multiple potential interventions have been tested to protect the heart against myocardial ischemia/reperfusion (MIR) injury. Remote ischemic conditioning (RIC), an endogenous cardioprotective approach, could markedly improve cardiac function post-myocardial ischemia injury. In this study, we aimed to assess the effects of RIC on cardiac sympathetic nerve innervation and metabolism in the association with Chondroitin sulfate proteoglycans (GSPG).

Methods

Transient myocardial ischemia (30 min) is induced by ligation of the left anterior descending coronary artery ligation (LAD) in male Sprague Dawley rats (250-350 g), in vivo cardiac [^{11}C]mHED and 2-[^{18}F]FDG PET scans were performed at 14 days after ischemia. Remote ischemic preconditioning (RIPerc) was induced by three cycles of five-minute-long unilateral hind limb ischemia and intermittent five minutes of reperfusion during LAD occlusion period. The quantitative parameters were quantified in parametric polar maps. This standardized format facilitates the regional radioactive quantification of parameters in deficit regions to remote areas. The ex vivo radionuclide distribution was additionally identified using autoradiography. Myocardial neuron density and GSPG expression were assessed by immunohistochemistry.

Results

There was no significant difference in the metabolism-defected to the remote activity ratio ($44.6\pm4.8\%$ vs. $45.4\pm4.4\%$) between control rats (MIR) and treated (MIR+RIPerc) rats ($P>0.05$). Additionally, the mean nervous activity of denervated myocardium activity was significantly elevated in rats with RIPerc coupled with reduced denervated myocardium size compared to the rats MIR group ($35.9\pm7.1\%$ vs. $28.9\pm2.3\%$ of the left ventricular (LV) remote area ($P<0.05$). These findings were associated with preserved LV systolic function and a significant reduction in GSPG expression in the myocardium.

Conclusion

RIPerc presented the effect on cardiac sympathetic nerve innervation following ischemia, but there is no significant effect on myocardial metabolism. A long-term outcome study is warranted.

Introduction

Myocardial infarction (MI) followed by adverse cardiac remodeling and heart failure (HF) is a leading cause of mortality and morbidity ^[1, 2]. Despite the robust development of early revascularization and pharmacological therapy, adverse cardiac remodeling leads to poor clinical outcomes in patients with MI ^[3]. Therefore, novel therapeutic strategies that effectively mitigate or reverse adverse post-infarcted cardiac remodeling and dysfunction remain challenging. Besides cardiomyocytes death due to acute MI, transmural MI damages sympathetic nerve (SN) fibers running through the infarct zone and results in regional sympathetic denervation in the heart ^[4, 5]. It is also known that the SN in the myocardium is more prone to suffer from ischemic insult than cardiomyocytes ^[6, 7].

Nerve injury causes the 'activation' of peripheral glia, the Schwann cells (SCs), whose repair-specific functions affect the whole microenvironment ^{[8] [9]}. These findings suggest that the local environment can manipulate SCs associated with nerves in injured organs to provide tissue-specific support. During regeneration of the heart muscle with its unique innervation, neuregulin-1 (NRG1) and Nerve growth factor (NGF) are highly upregulated in the injured/infarcted heart ^{[10] [11]}. Their regenerative effect has been primarily associated with cardiomyocyte proliferation and maybe nerve re-innervation, but NRG1 signaling may also be crucial for the proliferation, survival, motility, and differentiation of SCs following a myocardial injury such as MI ^[12]. In line with that, our group has demonstrated that remote ischemic preconditioning (RIPerc) markedly improved cardiac function and post-MI adverse remodeling and this was associated with an increase of cardiac NRG-1 expression in the rat model of myocardial ischemia/reperfusion injury (MIR) ^[13]. However, whether RIPerc alleviates or not cardiac SN innervation following MIR is largely unknown.

Regional quantification of cardiac SN function, in conjunction with an assessment of regional viability, is essential for investigating ischemia injury. Cardiac positron emission tomography (PET) utilizing 2-deoxy-2-[¹⁸F]fluoro-D-glucose (2-[¹⁸F]FDG) showed superior sensitivity for myocardial metabolism assessment. Of importance, PET with the ¹¹C-labeled norepinephrine analog [¹¹C]*meta*-Hydroxyephedrine ([¹¹C]*m*HED) enables non-invasive identification of local cardiac SN injury in patients with HF and ischemic cardiomyopathy ^{[14] [15] [16]}. Altogether, these reports indicate that the delay of cardiac SN innervation in infarcted and peri-infarcted tissue may be considered an essential component and surrogate marker of post-MI adverse LV remodeling and sudden cardiac death. Therefore, utilizing PET imaging and developing novel pharmacological or non-pharmacological approaches for boosting early innervation may significantly reduce cardiac mortality in patients with MI.

Methods

Animals

Male Sprague-Dawley rats (10-12 weeks old, 250-300 g, Department for Laboratory Animal Science and Genetics, Himberg, Austria) were used. The experimental protocol was approved by the Regional Ethics Committee for Laboratory Animal Experiments at the Medical University of Vienna and the Austrian

Ministry of Science Research and Economy (BMFW-66.009/xxx-WF/V/3b/2016), and conforms with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Myocardial ischemia and reperfusion in vivo

Myocardial ischemia was induced by ligation of the left coronary artery (LCA) for 30 min followed by 14 days of reperfusion as described previously in rats ^[13]. Briefly, rats were anesthetized by intraperitoneal injection of a mixture of Xylazine (4 mg/kg; Bayer, Germany) and Ketamine (100 mg/kg; Dr E. Gräub AG, Switzerland), intubated (14-gauge tube) and ventilated (9 ml/kg body weight, 75-85 stroke/min). To induce myocardial ischemia, the heart was exposed via a left thoracotomy and a ligature was placed around the LAD 2-3 mm away from the origin. Rectal temperature was measured and maintained at 37.5–38.5°C by a heated operating table. Myocardial ischemia was associated with the pallor of the myocardial area at risk and ST-elevation on ECG signal. Reperfusion was initiated following the 30 min of LAD occlusion by removal of the snare. RIPerc was induced by three cycles of 5 minutes of IR on hindlimb performed during myocardial ischemia as described previously ^[17]. Analgesia was initiated by intraperitoneal injection of Piritramide (0.1 ml/kg body weight) preoperatively and Piritramide in drinking water was applied as a postoperative analgesic regimen (2 ampules of Piritramide with 30 ml of Glucose 5% in 250 ml water).

Small Animal PET/CT Imaging

A dedicated small animal μ PET/ μ CT system (Inveon, Siemens Medical Solutions Inc., Erlangen, Germany) was employed. All animals were maintained under anesthesia throughout the imaging acquisition. The ECG gated 30 min dynamic [^{11}C]mHED PET (40-50 Mbq) and 60 min dynamic insulin-stimulated 2-[^{18}F]FDG PET (30-40 Mbq) acquisition was conducted meanwhile at tracer injection. All data were acquired in three-dimensional (3D) list-mode and sorted into a 3D sinogram of 16 frames per cardiac cycle. The sinogram was reconstructed into a 128 x 128 x 95 voxel image by 2D-filtered back-projection image reconstruction using a ramp filter with the Nyquist limit (0.5 cycles/voxel) as the cut-off frequency. The voxel size equaled 0.43 x 0.43 x 0.80 mm. Data were normalized and corrected for randoms, dead time, and decay. A 10 min attenuation correction transmission scan was additionally performed before the μ PET scan.

Imaging analysis

Late uptake defined the LV with bottle-brush sampling. Late myocardial uptake was averaged from 4 frames of data for each imaging 15 to 30 min after [^{11}C]mHED injection and 45 to 60 min after 2-[^{18}F]FDG injection. Using the volumetric sampling of the cardiac PET/CT scans, we defined myocardial radioactivity LV sectors and semi-automatically constructed radioactivity into cardiac metabolism and nerve polar maps. Each polar map was normalized to a region of interest (ROI) in the remote myocardium. The metabolic deficit area in the myocardium was defined as 2-[^{18}F]FDG uptake $\leq$ 60% of maximum activity. LV myocardium with nerve denervation was considered by a threshold of $\leq$ 60% of the

maximum activity in the polar map. [^{11}C]mHED retention in the denervated segment was determined. Myocardial ROIs were drawn at the mid-ventricular level, the blood pool was measured at the left atrial cavity. All μPET images were analyzed using the MunichHeart/NM software package.

Autoradiography

Ex vivo accumulation of myocardial [^{11}C]mHED was assessed using 20 μm sections of sample tissues with a digital autoradiography system (Cyclone® Plus Storage Phosphor System; PerkinElmer). Myocardial area of tracer uptake defect was determined by a threshold of 50% of maximum myocardial activity in midventricular slices; the radioactivity values of the placed ROIs were drawn on short-axis slices and then were expressed as background-corrected photostimulated luminescence units per area. Measured values were normalized to account for differences in the amount of tracer injected. Then the uptake activity of the defect area was calculated as percentages in relation to the remote area on the short-axis slice.

Assessment of LV hemodynamic in vivo

Next to $\mu\text{PET}/\mu\text{CT}$ Imaging, LV hemodynamic function was invasively monitored as described previously [18]. In brief, LV hemodynamic parameters were invasively measured. Rats were anesthetized by intraperitoneal injection of a mixture of Xylazin (4 mg/kg; Bayer, Germany) and Ketamin (100 mg/kg; Dr E. Gräub AG, Switzerland), intubated and ventilated. The chest was opened and a microtip catheter (SPR-409, 2F, Millar Instruments, Houston, USA) was gently inserted into the LV chamber. Hemodynamic parameters such as LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and heart rate (HR) were continuously recorded on Labchart (v7.3.2, Powerlab System (8/30, both AD Instruments, Spechbach, Germany) connected to a Bridge Amplifier (Bridge Amp, ADInstruments, GmbH, Spechbach, Germany) and a Transducer Control unit (Model TC-510, Millar Instruments Inc., Houston, Texas, USA).

Histology and immunochemistry

Formalin-fixed paraffin-embedded LV cardiac tissue sections were hematoxylin and eosin (HE) stained. Streptavidin-biotin immunostaining for CD68 (1:100; mouse monoclonal, ED1, ab31630, Abcam, Cambridge, MA, USA), Tyrosine Hydroxylase (1:100; rabbit monoclonal, EP1533Y, Abcam, Cambridge, MA, USA), Chondroitin Sulfate proteoglycan (CSPG; 1:500; mouse monoclonal, clone Cat-301, MAB5284, Merck, Darmstadt, Germany) were used as described previously [18]. Briefly, primary antibodies were detected with appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and horseradish peroxidase (HRP) conjugated streptavidin (Dako, Glostrup, Denmark), developed with 3,3'-diaminobenzidine (DAB) (Vector Laboratories), counterstained with hematoxylin, dehydrated and mounted in DPX (Merck, Darmstadt, Germany). Tyrosine Hydroxylase was developed with 3-Amino-9-ethylcarbazole (AEC) under visual control, counterstained with hematoxylin, and mounted with Aquatex (Merck, Darmstadt, Germany). Digitalized images were generated with a Nikon Eclipse 80i (Tokyo, Japan) microscope.

Statistics

Parametric data are presented as mean $\pm$ SD. Uptake variables of animals with MIR and RIPerc were compared with unpaired Student t-tests (continuous data). All statistical analyses were performed with commercial software (SPSS 24). A value of P less than 0.05 was considered statistically significant.

Results

Animal

Experimental rats were allocated into the following groups; 1) MIR (n=14) and 2) RIPerc with three cycles of 5 minutes of IR of a hindlimb performed during myocardial ischemia (MIR+RIPerc, n=15). Sham-operated animals (Sham, without LAD occlusion; n=6) served as controls. Fourteen days after the reperfusion, μ PET scans were performed (function, viability etc), hemodynamic function was performed 2 days after μ PET analysis *in vivo* and heart were taken for radioassay and immunohistochemistry, respectively.

[^{11}C] m HED Imaging

The metabolism (2-[^{18}F]FDG uptake) presenting average myocardial metabolism-deficit activity ratio was $44.6 \pm 4.8\%$ of the LV remote area in rats (MIR) vs $45.4 \pm 4.4\%$ of the LV remote area in rats (MIR+RIPerc) ($P > 0.05$). Additionally, the mean nervous activity ratio ([^{11}C]mHED uptake) of denervated myocardium activity was significantly elevated in rats with RIC in comparison to the MIR group ($35.9 \pm 7.1\%$ vs. $28.9 \pm 2.3\%$ of the LV remote area ($P < 0.05$)). (Fig. 1)

Furthermore, the animal cardiac polar map determined that the metabolism-deficit (2-[^{18}F]FDG uptake) area was $15.26 \pm 2.47\%$ of the total LV in rats (MIR) vs. $14.67 \pm 4.74\%$ of the total LV in rats (MIR+RIPerc) ($P > 0.05$). Additionally, the denervated myocardium area of the LV% was significantly increased in rats with MI in comparison to MIR+RIPerc ($27.78 \pm 6.62\%$ vs. $19.52 \pm 5.3\%$ of the LV in rats; $P < 0.05$) (Fig. 2). Quantitative cardiac polar map of viability (2-[^{18}F]FDG), and sympathetic innervation [^{11}C]mHED PET images from 2 representative subjects (MIR vs MIR+RIPerc) are presented in Figure 1.

Representative autoradiography was shown in Figure 3, ex vivo [^{11}C]mHED uptake ratios to the remote region were 0.29 ± 0.11 (MIR) and 0.37 ± 0.08 (MIR+RIPerc) ($p < 0.01$). (Fig. 3)

Immunohistochemistry

There is a more prominent reduction in TH-positive cells in MIR rats in comparison to RIPerc animals (Fig. 4). The reduction of cardiac neurons after MIR was shown to link a transient overexpression of CSPG in the animal hearts^[19]. Therefore, we assessed immunohistochemistry staining for CSPG in Sham, MIR and MI+RIPerc cardiac tissue samples. In comparison to Sham animals, the expression of CSPG was markedly increased in cardiac tissue samples from MIR rats (Fig. 5) particularly; its expression was higher in the infarcted area and border zone. In contrast, in rats treated with RIPerc, we observed that the CSPG expression was similar to the Sham group (Fig. 5). In addition, immunohistochemistry was

performed to evaluate the influx of CD68+ macrophages. No CD68+ cells were detected in the Sham group (Fig. 5). In contrast, high numbers of CD68+ macrophages were present in the infarcted myocardial tissue of the MIR group, whereas RIPerc was associated with a reduced influx of CD68+ macrophages (Fig. 5)

Left ventricular hemodynamic function

Left ventricular hemodynamic function was invasively monitored and analyzed the HR, LVSP, LVEDP, and $\pm$ LVdp/dtmax. No differences between the groups were observed in HR (Table 1). However, decreased LVSP, increased LVEDP, and reduced peak velocities of pressure change during isovolumic contraction (+dP/dt) and isovolumic relaxation (-dP/dt) in rats with MIR were observed in comparison to the sham group ($P<0.01$, respectively). These parameters were markedly improved by RIPerc (Table 1).

Table 1. Left ventricular hemodynamic parameters

Parameter/Group	Sham-OP	MIR	MIR+RIPerc
n	4	5	6
LVSP (mm Hg)	117 $\pm$ 5	83 $\pm$ 5 ^{***}	95 $\pm$ 8 [#]
LVEDP (mm Hg)	2.46 $\pm$ 0.50	5.70 $\pm$ 1.49 ^{***}	4.18 $\pm$ 0.63
+dP/dt (mm Hg -1)	7074 $\pm$ 564	4255 $\pm$ 380 ^{***}	5139 $\pm$ 588 [#]
-dP/dt (mm Hg -1)	-5879 $\pm$ 478	-3125 $\pm$ 873 ^{**}	-3827 $\pm$ 669
HR (bpm)	258 $\pm$ 28	257 $\pm$ 40	244 $\pm$ 28
LVSP: left ventricular systolic pressure; LVEDP: left-ventricular end-diastolic pressure; (+/-)dP/dt: peak velocities of pressure change; HR: heart rate. Values are mean $\pm$ SD; **p<0.01 and ***p<0.001 Sham vs MIR, and [#] p<0.05 MIR vs MIR+RIPerc.			

Discussion

Alterations of cardiac nerve innervation in cardiac pathophysiological diseases have been increasingly noticed. Nevertheless, a therapeutic approach to reduce cardiac sympathetic dysfunction is limitedly available.

Cardiac sympathetic and parasympathetic innervation plays multiple roles in cardiac physiology and pathophysiology. There is large body of evidence that selective parasympathetic, eg. vagus nerve stimulation during acute myocardial infarction protects the heart against myocardial IR-injury and chronic stimulation of the vagus nerve has also benefit on post MI remodelling and chronic heart failure, respectively [20] [21] [22]. However, large clinical trials failed to show similar benefits in patients with congestive HF.

On the other hand, sympathetic hyperinnervation or denervation in the myocardium are connected to the progression of cardiac dysfunction [23] [24]. Sympathetic denervation typically occurs in the infarcted myocardium and is associated with sudden cardiac death [25] [26]. Impaired innervation was also demonstrated in non-infarcted myocardium in patients with ischaemic and dilated cardiomyopathy [23]. Factors affecting SN integrity in infarcted and remote myocardium are largely unknown. Nevertheless, a recent pioneering study by Siebert *et al* [27] demonstrated that CSPG expression in the infarcted myocardium delays the sympathetic innervation following myocardial infarction. Moreover, microvascular perfusion abnormalities, even in the absence of epicardial coronary artery disease, may also relate to sympathetic dysfunction [23]. Therefore, novel non-invasive methods are urgently needed to visualize cardiac sympathetic (re)innervation in cardiovascular disease and find novel therapeutic approaches to improve cardiac sympathetic dysfunction.

Multiple shreds of evidence demonstrated that a portion of dysinnervated but viable myocardium is essential of cardiac sympathetic homogenous denervation with scar attenuates cardiac remodeling, improves cardiovascular function, as well as sympathetic denervation at myocardial scar may effectively reduce arrhythmia burden [28] [29] [30] [31]. In contrast, denervated but viable myocardium adjacent to infarcted myocardium may be particularly arrhythmogenic and prone to induce ventricular fibrillation and substantial risk for sudden cardiac death [22]. Thus, identifying nerve denervation in viable myocardium is crucial in patients with ischemic cardiomyopathy and may also provide surrogate markers for assessing the risk for sudden death. Cardiac molecular imaging allows for the specific identification of synaptic biomarkers in the cardiac autonomic nervous system.

The present study investigated the therapeutic effect of remote ischemic conditioning on cardiac sympathetic nerve re-innervation following myocardial infarction by quantitative *in vivo* imaging. Previous studies have investigated the cardioprotective signaling of RIC against acute MIR-injury and preventing cell death of cardiomyocytes [32] [33]. Moreover, Gho *et al.* demonstrated for the first time that the ganglion blocker hexamethonium abolishes cardioprotection established by RIC, suggesting the functional importance of neural (autonomic) pathways mediating this phenomenon [34]. However, evidence is lacking of whether RIC acts on SN activity and innervation in the subacute or chronic phase of MI. Therefore, we first assessed ischemia-mediated SN degradation and myocardial viability defection in a rat model of MIR. Interestingly, RIC presented cardiac protection against sympathetic denervation but has no protecting effect on viable myocardial stunning. These findings demonstrate that the SN might be more susceptible to ischemia injury than the myocardial cells. The remaining viable myocardium may preserve elevated SN fibers under remote ischemic conditioning and substantially preserve cardiac function. In line with that, we found that the RIC group showed significantly higher LVSP and a tendency to improve diastolic dysfunction (LVEDP) compared to the MIR group. Of importance, sympathetic denervation is strongly associated with microvascular dysfunction, which is the basis of the progression of diastolic dysfunction [23] [35]. Therefore, improving cardiac sympathetic dysfunction and re-innervation following MI may have potential clinical benefits in patients suffering from ischemic heart disease.

However, further studies need to clarify the interaction of RIC, microvascular dysfunction and sympathetic dysfunction /denervation in humans.

During acute myocardial ischemia, damage to cardiac sympathetic neurons results in the release of norepinephrine into the myocardial interstitial space^[36]. It has been recognized that the extent of denervation exceeds infarction following coronary artery occlusion. The neuronal damage could be transient or irreversible, depending on the severity and duration of ischemia, and reversible SN dysfunction has been reported but is slow in patients after myocardial ischemia^[6, 23, 25].

Chronic elevation of SN activity in the myocardium is a key component of the altered signaling pathways that accompany cardiac remodeling, increasing the risk for sudden cardiac death resulting from hypertension and acute MI^[24, 25]. However, the dysregulation and a decrease in SN activity play a pivotal role in impaired repair and regeneration response following insults of cardiac injury^[37]. In addition, a more recent study by Gardner *et al*^[19] suggests that early SN-reinnervation after MI reduced the risk for sudden cardiac death. Thereby therapeutic manipulation for boosting early SN innervation after MI may benefit LV remodeling, cardiac function and sudden cardiac death.

With injury or disease, an increase in CSPG expression is commonly observed close to lesioned areas^[27]. However, these CSPG deposits form a substantial barrier to regeneration and are primarily responsible for the inability to repair damage in the brain and spinal cord. A more recent study showed that CSPG present in the cardiac scar after MI prevents sympathetic innervation by binding the neuronal protein tyrosine phosphatase receptor σ (PTP σ) and downstream signaling pathway activation may increase the risk for sudden cardiac death^[19]. Interestingly, we also found that CSPG expression in the infarct and peri-infarct area was markedly increased in rats with MIR, but its expression was substantially decreased in RIPerc animals. This suggests that RIPerc may reduce the risk for sudden cardiac death via a mechanism involving the reduction in expression of CSPG and substantially boosting early sympathetic re-innervation post MI. In summary, sympathetic denervation in patients with ischemia and reperfusion has been extensively recognized, but the potential effect of reversible SN dysfunction is less widely appreciated.

Limitations

Certain limitations of our study need to be acknowledged. Due to gated cardiac perfusion, scans were not performed to determine the cardiac contraction due to high kinetic positron energy and consequently extensive positron range for small animal images. In addition, our study was not aimed to assess cardiac fibrosis and cardiac function by echocardiography or MRI following MIR. However, we have shown that RIPerc reduces cardiac fibrosis and improves hemodynamic function in comparison with untreated rats^[13].

Conclusion

In this study, we found that remote ischemic preconditioning treatment boosted early innervation of cardiac SN following myocardial ischemia/reperfusion injury in association with the reduction in CSPG expression, whereas no net effects on the myocardial metabolism were observed.

Declarations

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Conflicts of Interest

The authors declare no conflict of interest.

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Figures

Figure 1

Polar maps of myocardial viability and sympathetic denervation were measured with 2-[^{18}F]FDG, and [^{11}C]meta-Hydroxyephedrine ([^{11}C]mHED), respectively. Representative animal MIR and MIR+RIPerc present the polar maps showing larger denervation volume (downregulated [^{11}C]mHED uptake) than infarct volume (defected 2-[^{18}F]FDG uptake). In contrast, the rat with MIR presents polar maps showing relatively increased denervation volume ([^{11}C]mHED) and uptake ratios than the rat with RIPerc.

Figure 2

The average, minimum and maximum cardiac uptake ratio of the tracer deficit areas (mean, min, max) to the remote area (SUVmean) as a percentage of the left ventricular short-axis slice. The average of $[^{11}\text{C}]m\text{HED}$ uptake defect was significantly higher in rats with RIPerc than rats with MIR (* $p < 0.05$). There is no significant difference in $2-[^{18}\text{F}]FDG$ uptake in the deficit areas between the two groups of rats.

Figure 3

Autoradiography of [^{11}C]mHED in representative rats with MIR and RIPerc. Relatively increased sympathetic innervation was found in the defect area of rats with RIPerc compared to rats with MIR (*).

Figure 4

Immunocytochemistry of tyrosine hydroxylase positive neurons in the myocardium. Representative images showing tyrosine hydroxylase staining in the anterolateral area of a sham rat (A), and the defect area in the left ventricular wall in MIR (B) and RiPerc (C) rats. TH-positive cells are extensively reduced in MIR rats after myocardial infarction compared to Sham as well as less reduction in RiPerc rats.

Figure 5

Effect of remote ischemic preconditioning on influx of CD68+ cells, and CSPG expression. Representative images from each group of rats, showing (up) CSPG and (down) CD68+ macrophages in the reperfused myocardium (magnification 200× and 400×, scalebar: 50 µm, respectively).