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ORIGINAL ARTICLE – OPEN ACCESS

Disease-Modifying Antirheumatic Drugs and Remote Ischemic Postconditioning Ameliorate for Myocardial Injury in Rats Under Cerebral Stroke

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ABSTRACT

Objective: Currently, there is uncertainty about the increased risk of myocardial infarction following ischemic stroke or transient ischemic attack and the method of risk management after the stroke. In this experimental study, we aimed to evaluate myocardial injury after inducing global cerebral ischemia in rats and assess their responses to the treatments with disease-modifying antirheumatic drugs (DMARDs) and ischemic postconditioning (PC).

Materials and Methods: Global cerebral ischemia was induced by occluding the bilateral common carotid arteries for 20 minutes and subsequently reperfusing them. Thirty-two Wistar rats were divided into 4 treatment groups (n=8 for each group). Group I received 7 mg/kg/day infliximab immediately and at 6 hours after the stroke and group II received 10 mg/kg/day leflunomide immediately and at 6 hours after the stroke. In group III, the skeletal muscle in the limbs was clamped for 180 minutes immediately after the stroke and was reperfused for 120 minutes. Group IV was sham-operated and received saline immediately and at 6 hours after the stroke. Myocardium tissue samples were collected for histopathologic assays and to create hypoxia-induced tissue oxidative markers.

Results: We found that apoptosis and nucleus loss in the myocardium were significantly decreased after the administration of infliximab, leflunomide, and remote ischemic PC. Necrosis in the myocardium and cardiac malondialdehyde (MDA) level were also significantly decreased after treatment with remote skeletal muscle PC.

Conclusion: Our findings demonstrated that remote ischemic PC and DMARDs were protective against cerebral ischemia/reperfusion injury. They acted by mobilizing the endogenous adaptive mechanisms in the myocardium and inhibited oxidative stress by increasing the activity of antioxidant enzymes.

Keywords: Ischemia-reperfusion injury, myocardial infarct, cerebral stroke, disease-modifying antirheumatic drugs

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INTRODUCTION

Stroke and myocardial infarction (MI) have similar risk factors and underlying mechanisms. Compared to the general population, stroke patients in particular are at an increased risk for subsequent MI. However, patients who have had a stroke and are asymptomatic for related cardiac diseases are not routinely examined for potential coronary artery disease (CAD).

Several studies indicated that patients who had suffered a transient ischemic attack (TIA) or stroke were at high risk for CAD. The risk of MI is 1% in stroke patients without a history of CAD, while it is 3.6% in those with a history of CAD. The estimated risk of MI after stroke is independent of the etiology of ischemia, however, the male sex, hypertension, history of CAD, and peripheral artery disease were reported to double the increase of causal MI risk (1). The American Heart Association and the American Society of Stroke Association suggested that patients with stroke should undergo individual cardiovascular scoring and risk assessment (2). However, there is no reliable method to estimate the exact risk for MI that may occur after a stroke.

In cerebral ischemia, the release of various local and systemic cytokines, particularly tumor necrosis factor-α (TNF-α), is induced, which leads to the release of more cytokines, upregulates endothelial adhesion molecules, causes migration of leukocytes, and eventually induces endothelium to reach the prothrombotic stage (3). Although some studies have shown that disease-modifying antirheumatic drugs (DMARDs), such as leflunomide and TNF-α antagonists, may adversely affect cardiovascular diseases because of their adverse effects on blood pressure and lipid profile (4, 5), recent studies have suggested that TNF-α antagonists may reduce the risk of CAD, which is attributed to the direct action of TNF-α inhibition on the atherosclerotic process (6, 7). The question of whether the new DMARD group agents have an impact on CAD remains unanswered.

Ischemic postconditioning is a new method of providing protection to the tissues against a hypoxic episode. It has been reported that experimental animals that have been exposed to the short-term hypoxia attacks may be protective in the ischemic tissue injury models, since short-term hypoperfusion presented a protective effect

against long-term ischemic injury (8). Previous studies have shown that ischemic postconditioning reduces the size of the infarct by preventing the formation of free radicals and slowing apoptosis (9). Cardiac ischemia includes myxoid changes in the myocardium, such as neutrophil/leukocyte infiltrations among muscle fibers, necrosis, and subsequent fibrosis (10).

Although the patients with TIA or stroke were defined as high-risk population for CAD, their estimated risk for MI or the relationship for acute MI that may occur after stroke is not clear. Evidence from data in experimental studies is essential before these trials are conducted on humans. Hence, in this study, we aimed to evaluate the myocardial injury after performing bilateral common carotid artery occlusion in rats. We analyzed the relationship between ischemic stroke and acute MI in rats and assessed their responses to the treatments with DMARDs (infliximab, leflunomide) and ischemic postconditioning.

MATERIALS and METHODS

Animals

A total of 32 adult male Wistar Albino rats weighing 200–300 g were included in the study. The rats were housed and maintained at 22°C, 60%±5% humidity, and a 12:12 hour light/dark cycle with free access to food and water ad libitum. Experiments in this study were conducted in strict accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. All protocols in this study were approved by the Local Animal Experimentation Ethics Committee (File No: 2018/11/02, Approval date: 20.11.2018).

Reagents

Leflunomide (Abdi Ibrahim, Turkey) was prepared as 10 mg/kg/day, mixed with drinking water, and given per oral in two doses in 1 ml volumes by oral lavage. Infliximab (Merck Sharp Dohme, Singapore) was prepared as 7 mg/kg/day, dissolved in sterile 0.9% saline, and administered intraperitoneally in two doses in 1 ml/kg volumes.

Experimental Groups

Thirty-two rats were randomly divided into 4 groups, with 8 rats per group. The model of global cerebral ischemia was induced by the occlusion of bilateral common carotid arteries as previously described by Zhou et al. (11). Rats were placed on the operation table in the supine position after being anesthetized with ketamine HCl (80 mg/kg) and xylazine (10 mg/kg). After a superficial microdissection with a midline incision, a deep microdissection was performed toward the common carotid artery. Both common carotid arteries were exposed via the midline incision in the neck and were temporarily clipped for 20 minutes with cross-clamps. Following the occlusion, the clips were removed to restore the blood flow for recirculation and reperfusion was allowed for 2 hours.

Group I (Inflx) received 7 mg/kg/day infliximab intraperitoneally in two doses, immediately and at 6 hours after reperfusion by carotids.

Group II (Lef) received 10 mg/kg/day leflunomide per oral in two doses, immediately and at 6 hours after reperfusion by carotids.

Group III (PC) underwent ischemic postconditioning that was formed by clamping the unilateral lower limb for 180 minutes immediately after reperfusion by carotids. Lower limb ischemia was induced by the application of a tourniquet that clamped on the upper third of the right leg as previously described by Ergün (12). The ischemic period of limb muscle was selected to be 180 minutes until the distal pulses of the compressed limb could not be taken. At the end of 180 minutes, the limb muscle reperfusion was allowed for 120 minutes. Reperfusion was verified by the reappearance of the distal pulses and normalization of the skin color. Limb ischemia was verified by measuring the elevated levels of creatine kinase and lactate dehydrogenase (LDH) for muscle destruction.

Group IV (control) was sham-operated, given saline intraperitoneally in two doses, immediately and at 6 hours after reperfusion by carotids.

Data and Sample Collection

After the experiments, the animals were sacrificed under general anesthesia. Heart samples were collected to analyze the levels of glutathione peroxidase (GPx) and malondialdehyde (MDA) in the cardiac tissue. Cardiac injury was evaluated by histopathological staining.

Histopathological Examination

The hearts were removed, immersed in fixative (10% formalin solution) for 24 hours, embedded in paraffin, subjected to an autotechnicon device (Leica ASP 300, Germany), and microsectioned at a thickness of 4 µm (Leica Microtome RM 2145, Germany). The myocardium samples were examined for the infarct-associated morphological changes (Olypus BX53 polarizing microscope, Germany).

Quantification of Myocardial Tissue Injury

An overall score of cardiac damage severity was semiquantitively assessed regarding apoptosis, necrosis, isolated lymphocyte increase, ovoid nucleus loss, congestion, vascular proliferation, and edema. The severity of myocardial damage was semiquantitatively scored as 0 (normal), 1.0 (mild), 2.0 (moderate), and 3.0 (severe) as previously described by Selcuk et al. (10).

Measurement of Glutathione Peroxidase (GPx) and Malondialdehide (MDA)

To evaluate the myocardial injury via hypoxia-induced oxidant markers, GPx and MDA were investigated in the cardiac tissue homogenates. Cardiac tissue samples were taken rapidly, washed in cold saline, and homogenized with cold 0.15 molar (M) KCl (10%, w/v). Tissue homogenates were centrifuged at 600 x g for 10 minutes at 4°C to remove the crude fractions. The supernatants were then centrifuged at 10,000 x g for 20 minutes to obtain the post-mitochondrial fraction. GPx activities were determined in the post-mitocondrial fraction. MDA levels in homogenates were determined using thiobarbituric acid according to the method by Buege et al. (13). GPx activity was measured using the method described by Paglia et al. with cumene hydroperoxide as a substrate (14). In this method, GPx activity was coupled with the oxidation of NADPH by glutathione reductase, which was followed by being spectrophotometrically analyzed at 340 nm at 37°C. Results were calculated using the extinction coefficient (6.22×10^3/M cm).

Table 1. Histopathologic evaluation of the myocardium

Variable	PC	Inflx	Lef	Control
Congestion	2.14±0.38	1.75±0.89	2.25±0.46	2.33±0.52
Vascular proliferation	0.14±0.38	0	0.13±0.35	0
Apoptosis	0.29±0.49[a]	1.00±0.76[a]	1.13±0.64[a]	2.00±0.63
Necrosis	0[b]	0.38±0.52	1.25±0.71	0.83±0.41
Isolated lymphocytes	0.29±0.49	0.13±0.35	0.13±0.35	0
Edema	0	0	0	0
Nucleus loss	0[c]	0.75±0.71[c]	0.50±0.76[c]	2.00±0.00

Data are expressed as mean±SD. [a,b,c]Significant p-values ($p<0.05$) vs control group. [a]Group PC ($p=0.003$), Group Inflx ($p=0.027$), and Group Lef ($p=0.03$); [b]Group PC ($p=0.003$); [c]Group PC ($p=0.001$), Group Inflx ($p=0.003$), and Group Lef ($p=0.003$); PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); SD: Standard deviation; vs: Versus

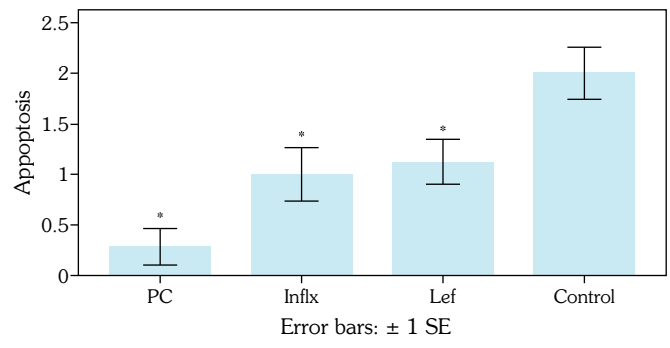

Figure 1. Apoptosis scores of groups

*Significance in the scores of apoptosis as compared to the control group. Group PC vs control ($p=0.003$), Group Inflx vs control ($p=0.027$), and Group Lef vs control ($p=0.030$). Data are expressed as mean±SD. PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); SD: Standard deviation; vs: Versus

Statistical Analysis

The data was represented in arithmetic mean and standard deviation. In order to apply the parametric analyses, the Kolmogorov–Smirnov test was used to determine whether the samples had normal distribution and whether the variances were homogeneous. For multiple groups, analysis of the variance test with a post-hoc Tukey's test for significance difference was used for normally distributed data. The Kruskal–Wallis test with the Mann–Whitney-U test under the Bonferroni correction was used for the analysis of non-normally distributed data. A p value of less than 0.05 were considered significant. The data was evaluated with a 95% confidence interval. The SPSS version 17.0 program was used for the statistical analysis.

RESULTS

Histopathologic Scores of the Myocardium After Inducing Cerebral Ischemia Reperfussion

The severity of myocardial damage was semiquantitatively scored as 0 (normal), 1.0 (mild), 2.0 (moderate), and 3.0 (severe) as previously described by Selcuk et al. (10).

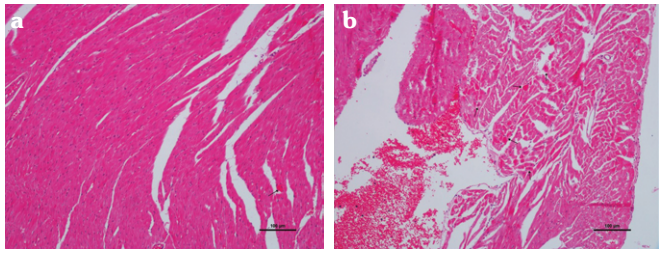

Figure 2. Appearance of apoptosis in myocardium in (a) Group PC and (b) control. H&E staining; magnification: x10

PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); H&E: Hematoxylin and eosin

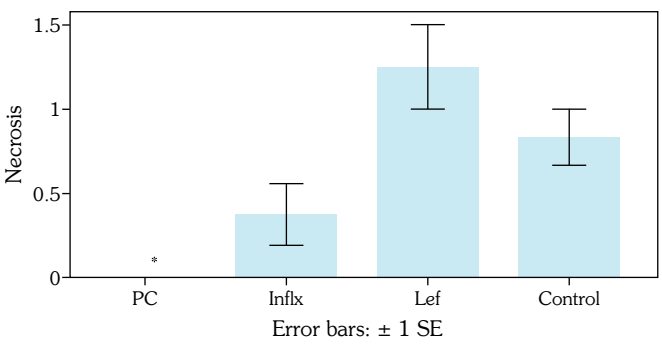

Figure 3. Necrosis scores of groups

*Significance in the scores of necrosis as compared to the control group. Group PC vs control ($p=0.003$). Data are expressed as mean±SD. PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); SD: Standard deviation; vs: Versus

As shown in Table 1, we found that apoptosis was scored 2.00±0.63 in the control group, 0.29±0.49 in Group PC, 1.00±0.76 in Group Inflx, and 1.13±0.64 in Group Lef (Fig. 1). Thus, apoptosis was found to be significantly higher in the control group than Group PC ($p=0.003$), Group Inflx ($p=0.027$), and Group Lef ($p=0.03$) (Fig. 2). Necrosis was scored 0.83±0.41 in the control group, 0.00 in Group PC, 0.38±0.52 in Group Inflx, and 1.25±0.71 in the Group Lef (Fig. 3). The score of necrosis was found to be significantly higher in the control group as compared to that of Group PC ($p=0.003$) (Fig. 4). Nucleus loss was scored 2.00±0.00 in the control group, 0.00 in Group PC, 0.75±0.71 in Group Inflx, and 0.50±0.76 in Group Lef (Fig. 5), which was significantly higher in the control group than in Group PC ($p=0.001$), Group Inflx ($p=0.003$), and Group Lef ($p=0.003$) (Fig. 6).

As shown in Table 1, congestion was scored 2.33±0.52, which was the highest (in the control group), and 1.75±0.89, which was the lowest (in Group Inflx). However, these values were not found to be significant. Vascular proliferation was insignificant among the groups with scores of 0.14±0.38 in Group PC, 0.00 in Group Inflx, 0.13±0.35 in Group Lef, and 0.00 in the control group. Isolated lymphocytes were scored 0.29±0.49, which was the highest (in Group PC) and 0.00 in the control group, although this was not found to be significant. Edema was scored as 0.00 in all the groups ($p>0.05$).

Glutathione Peroxidase (GPx) and Malondialdehide (MDA) Levels After Inducing Cerebral Ischemia Reperfusion

We evaluated the cardiac tissue levels of GPx and MDA in PC

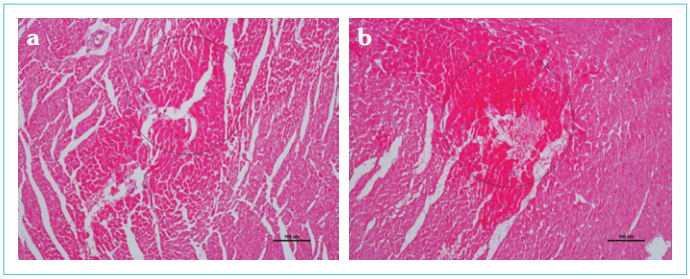

Figure 4. a, b. Appearance of necrosis in myocardium in (a) Group PC and (b) control. H&E staining; magnification ×10

PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); H&E: Hematoxylin and eosin

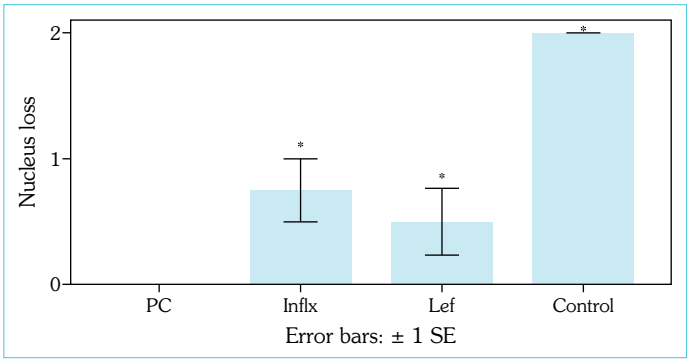

Figure 5. Nucleus loss scores of groups

*Significance in the scores of nucleus loss compared to the control group. Group PC vs control (p=0.001), Group Inflx vs control (p=0.003), and Group Lef vs control (p=0.003). Data are expressed as mean±SD. PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); SD: Standard deviation; vs: Versus

group, Infx group, Lef group, and compared with control rats. As shown in Table 2, we found that the mean level of GPx in the cardiac tissue of rats in Group PC was 983.20±247.27 nmol/g, 690.78±210.40 nmol/g in Group Inflx, 594.96±340.80 nmol/g in Group Lef, and 760.76±318.01 nmol/g in the control group. However, these values were found to be insignificant (p>0.05).

The mean level of MDA in the cardiac tissue of rats in Group PC was 73.17±12.98 nmol/g, 93.53±16.14 nmol/g in Group Inflx, 107.58±37.84 nmol/g in Group Lef, and 77.67±11.31 nmol/g in the control group. We determined that MDA in the cardiac tissue of rats in Group PC was significantly lower than Group Lef (p=0.039) (Fig. 7).

DISCUSSION

In this study, we evaluated the relationship between the ischemic stroke and acute myocardial injury in rats under global cerebral stroke, and their responses to the treatments with pharmacological agents (DMARDs; infliximab, leflunomide) and ischemic postconditioning. We found that the use of DMARDs and ischemic postconditioning were associated with a reduced risk of myocardial injury in rats under a cerebral stroke.

Myocardial ischemic conditioning provides an intervention that protects the myocardium from ischemia/reperfusion injury. Cardiac ischemic postconditioning, defined as three cycles of 30 seconds reperfusion and 30 seconds reocclusion at the immediate onset of

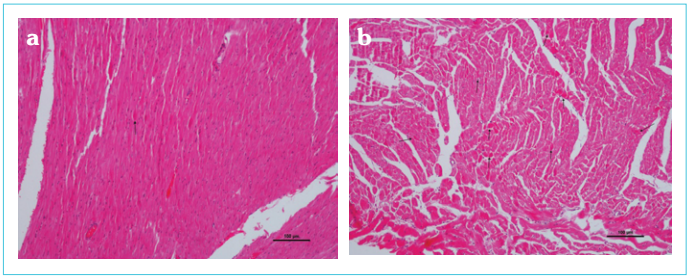

Figure 6. a, b. Appearance of nucleus loss in myocardium in (a) Group PC and (b) control. H&E staining; magnification ×10

PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); H&E: Hematoxylin and eosin

Table 2. Levels of glutathione peroxidase (GPx) and malondialdehyde (MDA) in the myocardial tissue

Variable	PC	Inflx	Lef	Control
GPx (nmol/gr)	983.20±247.27	690.78±210.40	594.96±340.80	760.76±318.01
MDA (nmol/gr)	73.17±12.98[a]	93.53±16.14	107.58±37.84	77.67±11.31

Data are expressed as mean±SD. [a,b,c]Significant p-values (p=0.039) vs Group Lef. PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); SD: Standard deviation; vs: Versus; GPx: Cardiac glutathione peroxidase level; MDA: Cardiac malondialdehyde level

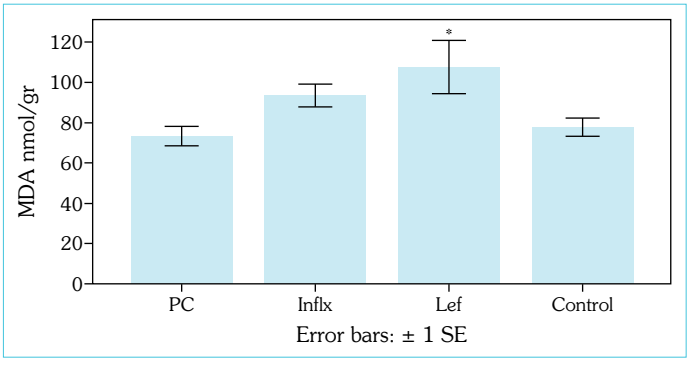

Figure 7. Cardiac MDA levels of groups

*Significance in cardiac MDA levels compared to Group Lef. and Group PC vs Group Lef (p=0.039). Data are expressed as mean±SD. PC: Postconditioning group (n=8); Inflx: Infliximab group (n=8); Lef: Leflunomide group (n=8); SD: Standard deviation; vs: Versus

reperfusion after 60 minutes of coronary occlusion, has been found to exert beneficial effects in reducing the infarct size by an equivalent amount (15). However, it may manipulate the atherosclerotic lesion resulting in the risk of coronary microembolization (16). Therefore, in our study, we induced postconditioning in the lower extremity skeletal muscle to avoid potential embolization of possible atherosclerotic lesions in the coronary vessels (11).

We found that necrosis in the myocardium and cardiac MDA levels were significantly decreased after postconditioning as compared to the control group. Prolonged ischemia can cause cell loss, however, recurrent short ischemia episodes in short terms in the distal skeletal muscle may have revealed an increase in the resistance to the

detrimental effects of the long-term ischemic condition in the heart (17), and thus improved the survival of the cells in the current study.

Nucleus loss and subsequent apoptosis were found to be significantly higher in the control group than in the treatment groups, as myocardial infarcts may decrease the cell survival due to pervasive cellular injury. The possible explanation may be that cytokines/chemokines have causal roles in ischemic pre- and postconditioning. The process of necrosis was found to be dependent on the activation of the receptor-interacting protein (RIP)1, RIP3, and a mixed-lineage kinase domain-like protein axis. This pathway is activated by TNF-α, which is also present in the cardioprotective signaling pathway of ischemic pre-conditioning (17). The association between TNF inhibitors and myocarfial infarction has been investigated previously and it has been demonstrated that TNF inhibitors offer a reduced MI risk, while some other studies found the risk to be similar when they were compared to DMARDs (6). In the present study, we found that apoptosis and nucleus loss cell were significantly decreased after TNF-α inhibitor infliximab and leflunomide as compared to the control group. Although TNF-α may reduce the infarct size by preventing or delaying apoptosis of cardiac myocytes and may have a homeostatic role in limiting the amount and duration of damage after an ischemic insult (18), neutralizing TNF-α with antibodies has also been shown to reduce the infarct size in murine models, which was similar to our findings (19). Thus, the definitive role of TNF-α- mediated signaling leading to either cell survival or necrotic cell death remains unclear. The possible explanation of our findings may be that blockade of TNF-α may modify the infarct size and ameliorate the severity of the injury via post-infarct remodeling (6).

There may be several possible explanations for a reduction in MI risk with DMARD therapy. One possible explanation may be a reduction in the MI risk due to the beneficial effects of DMARDs on cardiac risk factors. They have a steroid-sparing effect and reduce the systemic inflammation that can mediate a reduction in the risk of heart diseases (15). The protection was also demonstrated by the activation of a signal transducer and activator of transcription 3 (STAT 3) and mitochondrial K+ATP-channels (20). The neuroprotective effect of postconditioning has been abolished by the administration of N(ω)-nitro-L-arginine methyl ester (L-NAME) and LY-294002 [a phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) antagonist] in a previous work by Peng et al. (21), suggesting that ischemic conditioning protected the brain against global cerebral ischemia injury by up-regulating eNOS through the PI3K/Akt pathway. Coronary effluent of pre-conditioned hearts contains humoral factors, which reduce infarct size during early ischemic reperfusion in rats (22), which is partly mediated by PI3K/Akt signaling during ischemic reperfusion.

Although the cardiovascular effects of newer DMARDs are controversial, some data suggesting that DMARDs may increase blood pressure and lipids and other data showing the beneficial effects on cardiovascular risk was uncovered in the present investigation, where we found that ischemic postconditioning and the use of DMARDs were associated with a reduced risk of myocardial injury.

In conclusion, tissue protection against cerebral ischemia/reperfusion injury was achieved by mobilizing the endogenous adaptive mechanisms of the myocardium by leading an increased activity of antioxidant enzymes. Our histopathological observations were in accor¬dance with our biochemical results. We need to get more mechanistic insight into the postconditioning phenomena and identify the signaling pathways of cardioprotection in the myocardium in experimental animal models.

Ethics Committee Approval: All protocols in this study were approved by the Local Animal Experimentation Ethics Committee (File No: 2018/11/02, Approval date: 20.11.2018).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – DAA; Design – DAA; Supervision – DAA; Resource – DAA, MS, ÜK; Materials – DAA, MS, ÜK; Data Collection and/or Processing – DAA; Analysis and/or Interpretation – DAA; Literature Search – DAA, MS, ÜK; Writing – DAA; Critical Reviews – DAA, MS, ÜK.

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

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